VOLUME XXXII JULY, 1953 NUMBER 7

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

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The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 622 West 168th St., New York 32, N. Y.

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Acceptance for mailing at special rate of postage provided for in paragraph (d-2), section 34.40, P.L. & R. of 1948, authorized February 13, 1950

Entered as second-class matter September 16, 1931, at the Post Office at Lancaster, Pa., under the Act of March 3, 1879

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AN ABSTRACT OF THE SCIENTIFIC EXHIBIT

URINE AND THE GROUND SUBSTANCE

By Joseph Seifter, M.D.; A. H. Butt, M.D.; and E. A. Hauser, Ph.D.

Shown at the Annual Convention of the American Medical Association
New York City, June 1 to 5, 1953

Properties common to both urine and the ground substance of tissues were the subject of this exhibit. These common properties provide the basis for an understanding of a possible mechanism of action for hyaluronidase in the management of renal lithiasis.*

What Is Ground Substance?

"The ground substance is the mucoid cement material present between the cells and fibrils of the mesenchyme . . . The ground substance is practically everywhere in the body in the same way as mortar is present between the bricks of a house. The ground substance is both a barrier protecting cells from dangerous outside agencies and a conductor making available to cells the metabolites indispensable for cell life and removing waste products."

Significance of the Resemblance of Some of the Properties of Urine to Those of Ground Substance

"The mucopolysaccharide hyaluronic acid is a characteristic component of ground substance and is largely responsible for [its] state of viscosity . . . As a result of our studies on ground substance, we have come to look upon ground substance, not as an inert barrier, but as a dynamic material which participates in the spreading and transport of materials between cells. This spreading and transport activity is always enhanced by hyaluronidase and almost always inhibited or delayed by stress, ACTH and cortisone.

"Hyaluronic acid seems to play the same role in urine as it does in intracellular spaces in making possible the transport of metabolites and inorganic ions for elimination. Without the 'protective colloid' and dispersing action of hyaluronic acid, precipitation and crystallization occur.

"The most critical role played by hyaluronic acid is probably in the tubules where a tremendous concentration of urine is occurring and where precipitation in the absence of 'protective colloid' should occur in accordance with von Weimarn's law.

"The subcutaneous administration of an adequate dose of hyaluronidase results in an increase of 'protective colloids' in the urine. This is associated with an increase in total reducing substances and in glucuronic acid . . ."

"On the other hand, stress or the administration of cortisone or ACTH results in crystalluria that is indicative of suppression of 'protective colloid' action . . ."

Summary of Results Following Administration of Hyaluronidase for Renal Lithiasis

Group	Stone Formation Controlled	Stone Formation Slowed	Stones Smaller and Less Dense	Stone Formation Not Controlled
A—15 Cases Primary† Stones	9(60.00%)	3(20.00%)	1(6.67%)	2(13.33%)
B—21 Cases Secondary Stones	7(33.33%)	7(33.33%)	1(4.77%)	6(28.57%)

*The only hyaluronidase used in the study reported in this exhibit was the Wyeth brand, WYDASE®.

†No associated infection or mechanical obstruction. All other stones are considered secondary.

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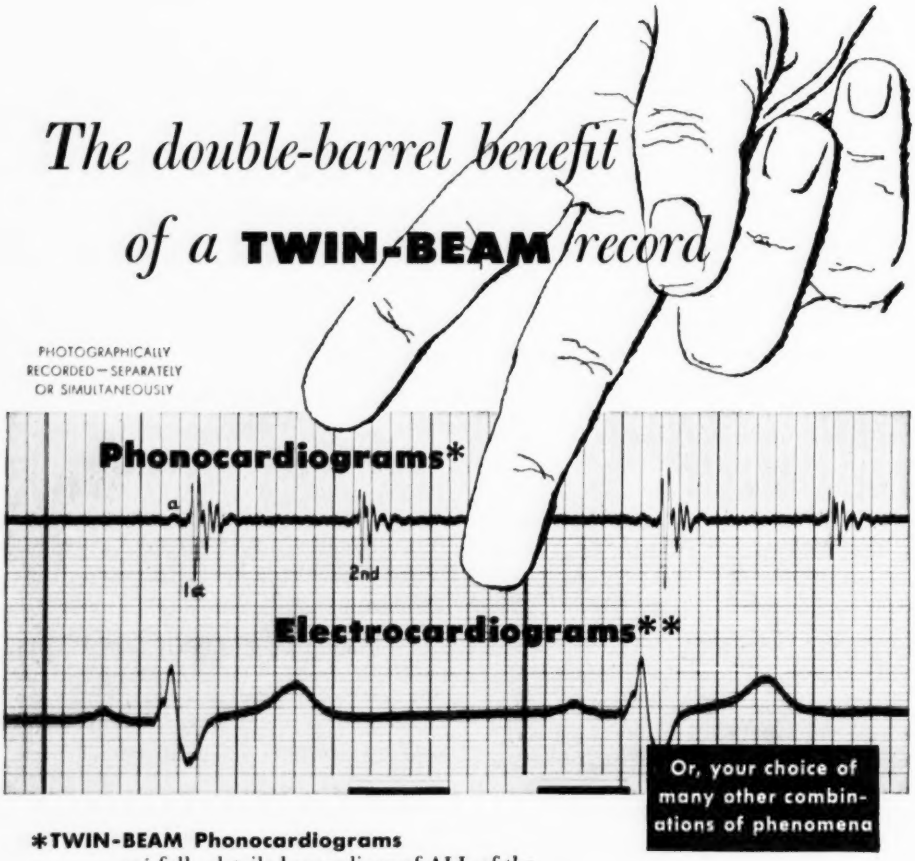

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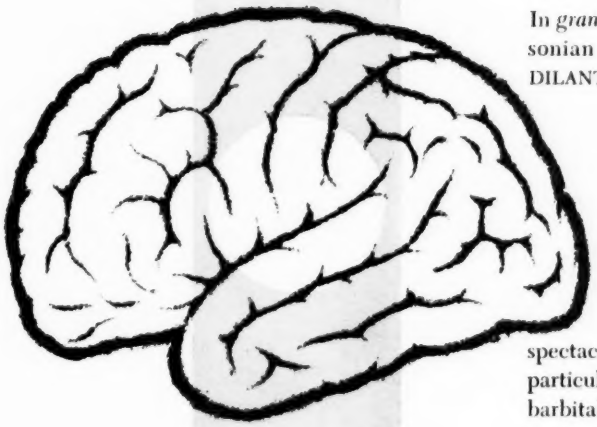

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(1) Krantz, J. C., and Carr, C. J.: The Pharmacologic Principles of Medical Practice, Baltimore, The Williams & Wilkins Company, 1949 (Reprinted 1950), p. 518. (2) *ibid*, p. 515. (3) Carter, S.: Epilepsy, in Conn, H. F.: Current Therapy 1952, Philadelphia, W. B. Saunders Company, 1952, p. 612. (4) Salter, W. T.: A Textbook of Pharmacology, Philadelphia, W. B. Saunders Company, 1952, p. 231.

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TABLETS

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LOBAR ALVEOLAR GAS CONCENTRATIONS: EFFECT OF BODY POSITION [1]

By C. J. MARTIN, FRANK CLINE, JR., AND HELEN MARSHALL

(From the Department of Physiology, Firland Sanatorium and the Department of Medicine, University of Washington, Seattle, Washington)

(Submitted for publication November 3, 1952; accepted March 11, 1953)

The concept that the composition of alveolar air may vary in space and time has occupied the attention of many investigators. Changes in ventilation and/or perfusion may alter the alveolar gas composition in normal lungs. The first published work on unequal ventilation in the lungs is usually attributed to Grehant in 1864. Recent studies (1–5) have involved measuring the concentration of a given gas in the expired air sampled at mouth level. Variation in gas concentration in the alveolar portion of expiration has been used as evidence for unequal intrapulmonary gas distribution. The probability of unequal pulmonary blood flow has been discussed (6–8).

The purpose of the present study is to (*1*) measure simultaneously the end-expiratory alveolar gas concentration within the lobar bronchus of the right upper lobe and the right lower lobe (or segments of the same), and (*2*) determine the effect of body position upon these concentrations.

METHODS

Subjects were normal volunteers or patients with normal lungs. All were ambulant and between the ages of 21 and 35 years. Distribution between the sexes was approximately equal. Under basal conditions, the subject was given a barbiturate, 0.1 gm. by mouth, and all but three subjects were given atropine sulphate, 0.41 mgm., subcutaneously thirty to forty minutes before the experiment. The larynx and pharynx were anesthetized with cocaine (5 per cent) in epinephrine (1:4000) as for routine bronchospirometry. A number 16 urethral catheter, from which the distal 4 cm. had been amputated, was introduced through the mouth into the trachea. Two radio-opaque poly-vinyl catheters (2 mm. OD, 1 mm. ID, 70 cm. long) were introduced through this catheter. A wire guide filling the lumen of each poly-vinyl catheter provided better control of the distal tip. The right upper lobe catheter had an 80 degree curvature in its distal portion, beginning within 2 cm. of the tip. After the small catheters were in the trachea, the urethral catheter was removed and the subject was placed on the fluoroscopic table. Under direct fluoroscopic vision, each catheter was guided into the selected lobar bronchus and the wire guide removed. After the subject was in position for approximately 15 to 20 minutes the first samples were taken. The subject was in each subsequent position 5 to 20 minutes before further samples were taken. Simultaneous samples from the right upper and right lower lobe bronchi were taken in pre-evacuated mercury sampling tubes (9), so constructed that the dead space of the catheter and tube could be cleared before the sample was taken. Gas was analyzed in duplicate in a Scholander 0.5 cc. gas analyzer. Samples were taken in the last one-third of expiration or in the expiratory pause. If either of two observers questioned the timing, the samples were discarded. The volume of each sample seldom exceeded 6 cc.

Samples were taken with the subject in the horizontal position, in 10 to 30 degrees Trendelenburg and upright. A motor driven fluoroscopic tilt table was used to change position. Sequence of positions used was varied. At the termination of the procedure approximately 1 cc. of contrast mixture was instilled through each catheter and roentgenograms were taken to localize the tip of the catheter.

RESULTS

Results in 13 of 21 subjects are presented in Table I. We were unable to catheterize the right upper lobe orifice in five subjects. Two others experienced syncope when upright and are not included. One male subject had an abnormal respiratory rhythm with an inspiratory pause. This was, in effect, a breath-holding experiment which is being studied further.

Eighteen simultaneous right upper and right lower lobe gas samples were obtained from 11 upright subjects (Table I). In each instance the end-expiratory oxygen concentration from the right upper lobe was higher than that from the right lower lobe. The mean oxygen difference was 2.136 Vol. per cent which is statistically significant by the paired samples method ($P < .001$). End-expiratory carbon dioxide concentration was higher from the right lower than the right upper lobe by

[1] This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service, in part by a grant from the Firland Health Grads.

TABLE I

Simultaneous upper and lower lobe alveolar air values in upright, horizontal and Trendelenburg positions

Subject	Barometer	Catheter	Upright				Horizontal				Trendelenburg			
			Volume %		R.Q.*	Minutes upright before sample	Volume %		R.Q.*	Minutes supine before sample	Volume %		R.Q.*	Minutes in Trendel. position before sample
			O_2	CO_2			O_2	CO_2			O_2	CO_2		
	mm. Hg													
J. K.	729.5	RUL	13.00	4.72	0.54		12.36	4.43	0.46		†			
		RLL	10.74	5.10	0.44	3	14.98	4.23	0.66	15				
B. E.	741.4	RUL	18.74	1.77	0.78		17.69	3.08	0.95		16.18	3.60	0.72	
		RLL	17.79	2.30	0.69	7.0	18.37	2.80	1.14	10	18.08	3.06	1.11	5
J. D.	738.9	RUL	13.87	5.10	0.68		15.61	4.60	0.84		14.19	5.21	0.73	
		RLL	‡			30	15.64	4.73	0.88	15	15.37	4.64	0.80	5
S. C.	730.0	RUL	18.08	2.35	0.80		14.43	4.87	0.71		16.16	4.01	0.81	
		RLL	16.86	3.11	0.73	16.0	14.75	4.66	0.71	15	16.17	4.19	0.86	10
E. S.	768.7	RUL	13.63	5.13	0.65		9.26	5.94	0.45		10.90	5.84	0.53	
		RLL	11.28	5.50	0.51	5	11.52	5.69	0.55	11	9.72	5.95	0.47	6
		RUL	13.88	5.31	0.71									
		RLL	10.68	6.13	0.54	14								
B. W.	744.57	RUL	14.42	4.85	0.70		13.02	5.36	0.63		13.09	5.53	0.66	
		RLL	12.56	5.31	0.58	15	13.20	5.25	0.63	15	12.23	5.55	0.58	12
		RUL	14.54	4.87	0.72									
		RLL	12.58	5.43	0.60	18								
W. R.	745.5	RUL	14.97	4.24	0.67		14.35	4.37	0.61		13.87	4.67	0.61	
		RLL	12.45	4.95	0.53	7	14.86	4.40	0.68	14	14.93	4.26	0.66	5
		RUL	17.05	3.42	0.86									
		RLL	14.48	4.38	0.63	18								
F. O.	746.24	RUL	15.87	4.37	0.84		13.18	5.34	0.64		13.34	5.40	0.66	
		RLL	§			14	14.45	4.73	0.68	9	§			10
D. H.	717.84	RUL	18.12	2.90	1.05		15.12	4.08	0.65		15.80	3.68	0.67	
		RLL	15.81	3.74	0.69	4	17.76	2.83	0.88	11	17.27	3.42	0.93	6
		RUL					16.42	3.79	0.81					
		RLL					17.22	1.52	0.36	12				
S. B.	756.14	RUL	15.14	4.99	0.84		13.52	5.38	0.68		†			
		RLL	13.71	5.16	0.67	11	13.70	5.14	0.66	6				
		RUL	15.62	4.88	0.91									
		RLL	13.56	5.16	0.65	13								
F. P.	752.63	RUL	17.03	3.56	0.90		11.03	5.32	0.48		†			
		RLL	14.53	4.29	0.62	8	15.34	4.44	0.76	12				
		RUL	17.58	3.29	0.99		12.99	4.86	0.56					
		RLL	14.83	4.18	0.64	18	15.45	3.75	0.64	31				
E. N.	758.21	RUL	14.73	3.81	0.56		15.46	4.83	0.86		10.20	4.77	0.39	
		RLL	12.70	4.25	0.46	5	16.06	4.66	0.95	10	13.34	4.48	0.54	15
		RUL	14.97	3.98	0.62		14.31	4.31	0.60					
		RLL	12.71	4.55	0.50	7	15.45	4.16	0.72	13				
H. R.	748.3	RUL	18.20	1.95	0.67		16.50	4.09	0.91		†			
		RLL	16.17	3.13	0.61	6	15.93	4.36	0.85	5				
		RUL	17.55	3.18	0.94		16.37	4.06	0.87					
		RLL	15.35	3.95	0.66	24	15.74	4.29	0.80	19				
R. S.‖	756.0	LUL	15.81	4.39	0.83		13.58	5.80	0.75		11.52	6.11	0.60	
		LLL	14.23	5.00	0.70	7	14.82	5.53	0.89	23	14.64	5.05	0.77	6

* Correction formula from Handbook of Respiratory Data in Aviation.
† Samples not taken in this position.
‡ Catheter plugged, no sample obtained.
§ Sampling tubes broken.
‖ All samples from the left, see text.

a mean of 0.634 Vol. per cent. This is highly significant ($P < .001$). In each of the paired samples the respiratory exchange ratio was higher in the right upper lobe bronchus. The mean difference in R.Q. (correction formula for alveolar air from the "Handbook of Respiratory Data in Aviation") is 0.176 ($P < .001$).

In the supine position (Table I) there were 17 simultaneous upper and lower lobe determinations on 13 subjects. In each instance, excepting one subject (H. R.), the end-expiratory oxygen concentration was higher in the right lower lobe than in the right upper lobe. The mean difference was 1.106 Vol. per cent and is significant ($P < .005$). The end-expiratory carbon dioxide concentration was higher in the right upper lobe than in the right lower lobe by a mean of 0.416 Vol. per cent ($P = 0.02$). The respiratory exchange ratio was lower in the upper lobe bronchus by a mean of 0.049 (not statistically significant).

There was no statistically significant difference between right upper lobe and right lower lobe end-expiratory gas concentrations in eight subjects in Trendelenburg position (Table I). End-expiratory oxygen concentration in the right lower lobe bronchus was higher than in the right upper lobe bronchus by a mean of 0.84 Vol. per cent ($P < 0.10$). End-expiratory carbon dioxide was higher in the lower lobe bronchus by a mean of 0.22 Vol. per cent. The R.Q. was higher in the right lower lobe bronchus by a mean of 0.104.

Left upper lobe and left lower lobe end-expiratory gas concentrations have been determined in one subject (Table I). This man had moderately advanced pulmonary tuberculosis on the right, with no evidence of disease in the left lung. These values are similar to those described above for the right lung. These results are not included in the statistical study.

One subject (H. R.) when supine showed higher end-expiratory oxygen values in the upper lobe than in the lower lobe. Carbon dioxide and R.Q. values were similarly opposite to those of other subjects in this position. This study was repeated after a two month interval at which time similar differences were obtained. We have no explanation for this deviation from the usual pattern in an apparently healthy woman.

While precautions were taken to prevent contamination, these may have failed in some cases. Low carbon dioxide tensions may indicate inspiratory contamination or hyperventilation. Hyperventilation was noted in some cases. However, in all cases the respiratory exchange ratio was higher in the upper lobe in the upright subject, an indication of a real difference between the two lobes in their ventilation-perfusion ratios.

Independent statistical analysis by Dr. Paul T. Bruyere, National Institutes of Health, agrees with the preceding statistical values. Dr. Bruyere used the analysis of variance criteria and contributed further information on the above studies. "Comparisons of successive determinations in the upright position made on seven subjects reveal no significant trends. Total change between determinations, rate of change per minute, and rate of change related to number of minutes upright before the first determination were all studied. Oxygen and carbon dioxide concentrations and respiratory quotient all showed small average increases, but the changes varied greatly from one subject to another. No significant relationship could be established between any one of the three measures of respiration (oxygen, carbon dioxide, and respiratory quotient) and any of the accessory factors recorded; pulse, respiration, catheter position, medication." (The "catheter position" in Dr. Bruyere's analysis refers to the position within the lobe and not to differences between lobes which did show statistical difference.)

DISCUSSION

Others have obtained samples of alveolar air from the bronchi of human subjects. Loewy and Schrötter (10) (1905) used a catheter with an inflatable rubber cuff at its distal end, obtaining alveolar air in equilibrium with mixed venous blood. There was no ventilation of the lung area sampled. Birath (11) used small catheters which permitted lung ventilation. Samples were obtained from the trachea and lower lobe bronchi of tuberculous patients with pneumothorax. We are now studying the effect of collapse procedures on alveolar air concentrations.

By directly measuring end-expiratory alveolar air from the lobar bronchi, we have shown regional differences to exist. These lobar differences can be markedly changed by body position. When each subject was upright, the carbon dioxide con-

centration was lower, the oxygen concentration was higher, and the R.Q. was higher in the right upper lobe than in the right lower lobe. These relationships were reversed in most subjects in the supine position. Whether this pattern is a result of ventilation and/or perfusion changes cannot be stated.

"Spot" sampling of alveolar air has been criticized as being unable to describe a value which varies throughout a wide range. The numerical values given for lobar alveolar gas concentrations are not absolute but we believe simultaneous sampling from upper and lower lobe bronchi provide comparable values for one period (time) in the expiratory cycle. Our results show the effect of body position upon comparable samples from different lobes (space). Rapid gas analyzers may solve this problem.

Haldane, Meakins and Priestley (12), studying shallow breathing, felt that the recumbent position resulted in more unequal ventilation than the upright position. Rauwerda (5) shared this view. If unequal ventilation is the cause of the lobar differences in R.Q. observed in this study, we cannot agree with this conclusion. We found greater differences in alveolar gas concentrations between lobes in the upright than in the supine subject. Only by assuming a change in pulmonary perfusion can these findings be consistent with more even ventilation in the upright subject.

Roelsen (6) believed unequal pulmonary perfusion as well as unequal ventilation to be present in normal man. Rahn (7) and Riley and Cournand (8) assume the existence of unequal pulmonary blood flow as well as unequal pulmonary ventilation. Thus "mean" or "ideal" alveolar gas tensions, as defined by these authors, provide a value compatible with the quantitative aspects of gas exchange. Rahn has shown that "mean" alveolar gas tensions and end-tidal air tensions measured at mouth level are similar in normal man. End-tidal gas values from lobar bronchi suggest the magnitude of variation in ventilation-perfusion relationships which is being described by a single value.

Dock's (13) theory on the apical localization of adult pulmonary tuberculosis has been confirmed in one respect. He believed that when man was upright, the pulmonary blood flow was decreased in the upper portion of the lung as a result of low pulmonary arterial pressure. Hence there would be little gas exchange between the alveoli and blood in the apex of the lung and high oxygen tensions would exist. However, the work of Scott, Hanlon, and Olson (14) in monkeys suggests that pulmonary blood flow itself is not the major factor in this apical localization. Their monkeys with a systemic to pulmonary arterial anastomosis showed extensive caseous lesions on the side of the anastomosis. Similarly on the side of a ligated pulmonary artery the same type of lesion occurred. The opposite lung showed the miliary type of tuberculosis seen in control animals. Rich and Follis (15) have shown that the oxygen content of inspired air markedly influences tuberculous lesions in infected rabbits and guinea pigs. As the oxygen concentration was lowered below 12 Vol. per cent progressive inhibition of tuberculous lesions occurred. The experiments of Scott, Hanlon, and Olson suggest that the caseous changes observed in operated monkeys are related to high oxygen tensions in the arterialized pulmonary tissue. In the present study we have shown that high oxygen tensions exist in the apex of the lung in the upright man. Differences such as we have described in the upper and lower lobe oxygen concentrations may favor the growth of the bacillus in the apex when man is upright. Similarly when the subject is on bed-rest with lowered oxygen tensions in the upper lobe, the bacillus is at a disadvantage for its oxidative needs.

CONCLUSION

1. Lobar differences in alveolar gas concentrations do exist.

2. Body position affects lobar gas concentrations in the following manner:

a) When upright, end-expiratory oxygen is higher and carbon dioxide is lower in the right upper lobe than in the right lower lobe. The R.Q. is higher in the right upper lobe.

b) When supine, the end-expiratory oxygen is higher and the carbon dioxide concentration is lower in the right lower lobe than in the right upper lobe. The R.Q. shows no significant difference between the two lobes. Apparently ventilation-perfusion relation-

ships are not as greatly altered in this position as in the upright position.

c) In the Trendelenburg position there is no statistically significant difference between the upper and lower lobe gas concentrations.

3. A factor in the apical localization of adult pulmonary tuberculosis may be the higher oxygen tension in the upper lobe in the upright man.

ACKNOWLEDGMENT

We are indebted to Florence Best and Sergey Politoff for technical assistance and to the many volunteers who made this study possible.

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THE EFFECTS OF THE CARBONIC ANHYDRASE INHIBITOR "6063" ON ELECTROLYTES AND ACID-BASE BALANCE IN TWO NORMAL SUBJECTS AND TWO PATIENTS WITH RESPIRATORY ACIDOSIS[1]

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(Submitted for publication January 13, 1953; accepted March 18, 1953)

INTRODUCTION

The effects of oral administration of sulfanilamide in man on acid-base metabolism have been reported previously (1, 2). Sulfanilamide produces a transient increase in urine pH, CO_2 content, sodium and potassium and a transient decrease in urinary ammonia. These urinary changes are associated with a fall in the serum CO_2 content and a rise in serum chloride. The inhibition of the enzyme carbonic anhydrase by sulfanilamide was described by Mann and Keilin (3). It is believed that the effects of sulfanilamide on electrolyte and acid-base balance are due to the inhibition of this enzyme in the cells of the renal tubules (4). In 1949, Schwartz (5) reported the diuretic effect of sulfanilamide in three patients with congestive heart failure. However, the toxicity of this drug appeared to limit its possible usefulness as a diuretic agent.

In 1950, Roblin and Clapp (6) described the preparation and properties of a series of heterocyclic sulfonamides. Their activity as potent inhibitors of the enzyme carbonic anhydrase *in vitro* was further investigated and amplified by Miller, Dessert and Roblin (7). The present study concerns some of the effects of one of these compounds "6063" (Diamox [2]) (2 acetylamino-1,-3,4 thiadiazole-5-sulfonamide) on electrolyte and acid-base metabolism.

METHODS

Two male patients (W. B., age 32 and M. W., age 30), with neurological disease (muscular dystrophy and cerebral palsy) but without evidence of cardiac, renal or pulmonary disease were studied. In addition, two male patients (W. M., age 52 and S. D., age 64) with long-standing pulmonary emphysema and well documented respiratory acidosis were also studied. The latter patients had no evidence of renal functional impairment; both had electrocardiographic evidence of cor pulmonale and had exhibited a mild degree of right-sided congestive failure in the past for which they had been digitalized. Neither had evidence of cardiac failure at the time of these studies.

The subjects under investigation were placed on a constant diet containing 5 mEq. of sodium per day and received, in addition, 50 mEq. of sodium per day in the form of NaCl capsules. There was a constant daily intake of protein, fat and carbohydrate. Water was permitted *ad lib.* Daily 24-hour urine specimens were collected under mineral oil with the use of a special funnel-like urinal designed to minimize the loss of gases. A small amount of thymol was used as a preservative and the urine collecting bottles were kept on ice at the patients' bedsides. Determinations were made daily of urine volume, specific gravity, pH, CO_2 (8), ammonia (9), chloride (10), sodium, potassium and phosphate (11). The CO_2 content of urine kept under the described conditions was found to be approximately 1 to 2 mM per L. lower than that of freshly voided acid urine. The CO_2 loss from alkaline urine was found to be negligible. Determinations of blood pH, and plasma CO_2 content (8), chloride (10), sodium and potassium were performed at intervals as indicated in Figures 2, 4, 6, and 8. Urine and blood pH were determined with the Beckman pH meter Model G. The pH determinations were performed at room temperature (approximately 25°C.). The blood pH values were corrected to 37°C. by subtracting 0.014 pH units per degree difference in temperature. The urine pH values were not corrected for temperature. Urine and blood sodium and potassium were determined on an internal standard flame photometer (12). The pCO_2 was calculated from the arterial pH and CO_2 content using the Henderson-Hasselbalch equation and a pK value of 6.1 for H_2CO_3. All blood samples were obtained from the brachial artery and were collected under mineral oil. During the experimental periods on "6063" blood samples were collected approximately two hours after the administration of the drug.

[1] Supported in part by the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, The National Heart Institute, and the Albert and Mary Lasker Foundation.

[2] Provided by Lederle Laboratories Division, American Cyanamid Co., New York City.

After a foreperiod of 12 to 15 days the patients were started on oral "6063"; initially 8 to 10 mg. per kg. body weight per day were given in four divided doses at six-hour intervals. In some cases the dose was later increased up to 18 mg. per kg. body weight per day.

RESULTS

Figures 1 to 8 show the changes in the blood and urine constituents for each patient. The table gives a summary of the results obtained in the urine studies.

Acute changes. During the first two days of administration of "6063" there was an increase in the urine pH and a 10 to 20 fold increase in the urine CO_2 content. There was also an increased urinary excretion of sodium and potassium (one to four fold) and a slight increase in urinary phosphate.

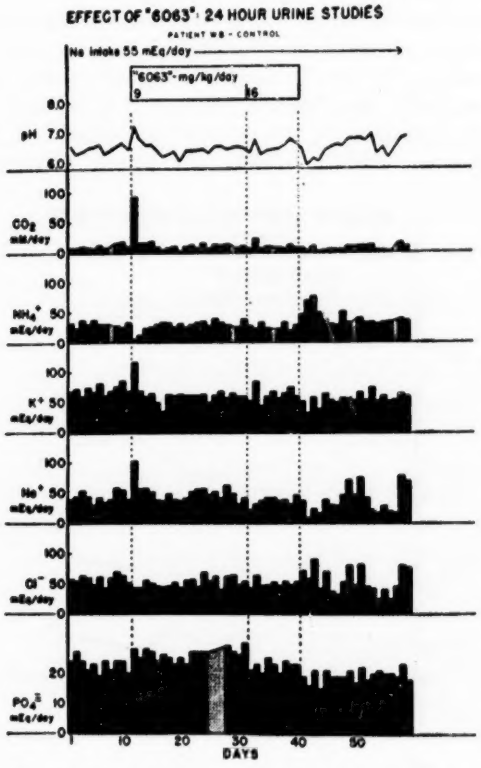

FIG. 1. DAILY 24-HOUR URINE CONSTITUENTS OF CONTROL PATIENT W. B. (MUSCULAR DYSTROPHY) DURING STUDY PERIOD

The stippled areas indicate days when determination was omitted for technical reasons.

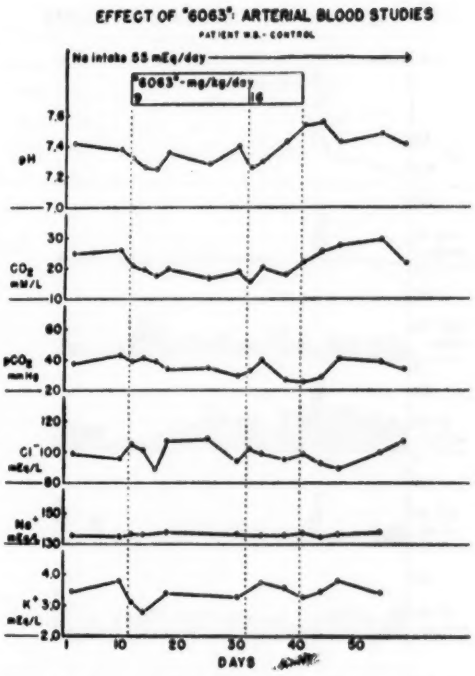

FIG. 2. ARTERIAL BLOOD STUDIES OF PATIENT W. B.

Urinary ammonia decreased about 50 per cent. There was no significant change in urine chloride. There was no significant change in urine volume. Consistent results were seen in all four patients.

One day after the drug was started there was a fall in arterial pH (0.1 to 0.2 pH units) associated with a fall in the plasma CO_2 content of 5 to 10 mEq. per L. In three of the four patients there was an associated rise in the plasma chloride concentration. During the first week of the drug therapy there was a fall in the plasma potassium concentration. There were no clinical symptoms of hypokalemia. There was no significant change in the plasma sodium concentration.

Changes with prolonged administration of "6063". After a maximum of three days the urine pH and electrolyte excretion returned to control values and continued unchanged for the remaining 25 to 55 days of drug administration. When the dosage was increased up to a maximum of 18 mg. per kg. per day there was no further change in the urinary constituents. These dosages are comparatively small; larger doses might have yielded different results.

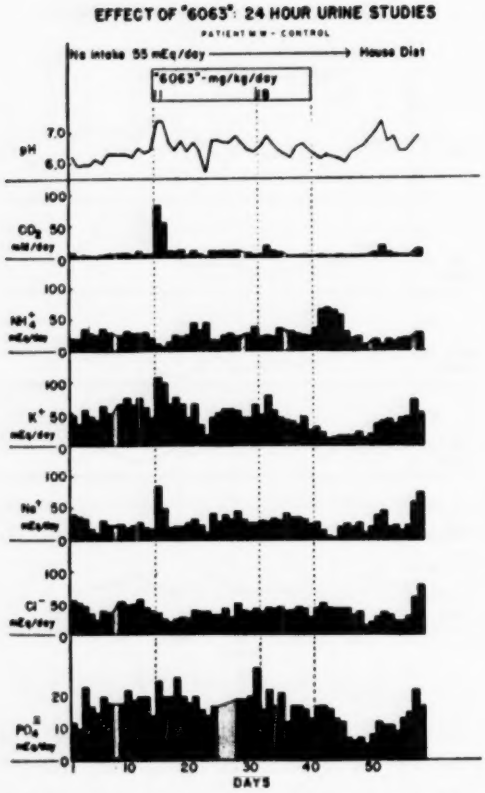

FIG. 3. DAILY 24-HOUR URINE CONSTITUENTS OF CONTROL PATIENT M. W. (CEREBRAL PALSY) DURING STUDY PERIODS

During the entire period of administration of "6063" the arterial CO_2 content was depressed 5 to 10 mEq. per L. below the control values in both groups. However, the arterial pH tended to return to control values, and in both patients with respiratory acidosis, where the pH had previously been low, it rose to normal several weeks after therapy was started. This was due to a fall in the arterial pCO_2. In the patients with respiratory acidosis the plasma chloride rose, in one case to normal. After the initial period of depression, the plasma potassium tended to return to control values. There was no change in the plasma sodium concentration.

Changes on discontinuation of "6063". For one to five days after the discontinuation of "6063," there was decreased urinary excretion of sodium with no significant change in urine chloride excretion. In one of the three patients studied in this period (M. W.), there was also a significant decrease in potassium excretion. For four to six days after cessation of "6063" therapy, there was increased urinary excretion of ammonia in all cases.

After the drug was discontinued the arterial CO_2 content in the control patients returned to normal in four to five days; the arterial pH rose to abnormally high values (7.55 to 7.56) in four to seven days. Three to four days later, the arterial pH had returned to normal. In the patients with respiratory acidosis the arterial CO_2 content rose to previous values in three to five days; the arterial pH fell to previous control values in five to seven days.

Clinical changes. The two control subjects were asymptomatic during the entire period of study. One patient (W. M.) (Figures 7 and 8), who had previously been comatose because of respiratory acidosis and who had required the daily use of a respirator, was maintained in an alert state outside the respirator for two separate periods while receiving "6063". Initially, the patient received

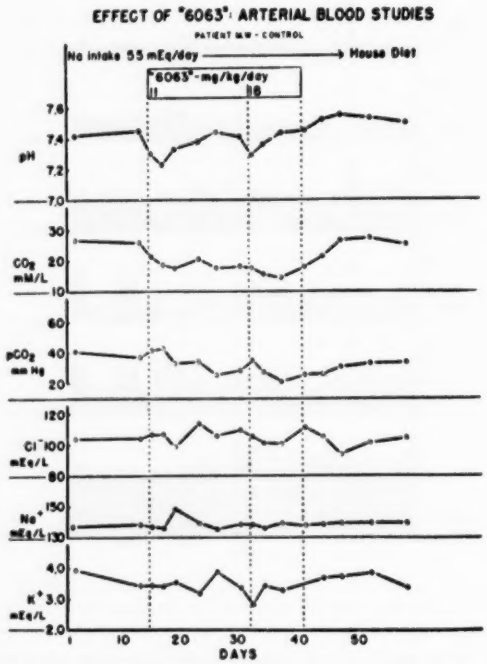

FIG. 4. ARTERIAL BLOOD STUDIES OF PATIENT M. W.

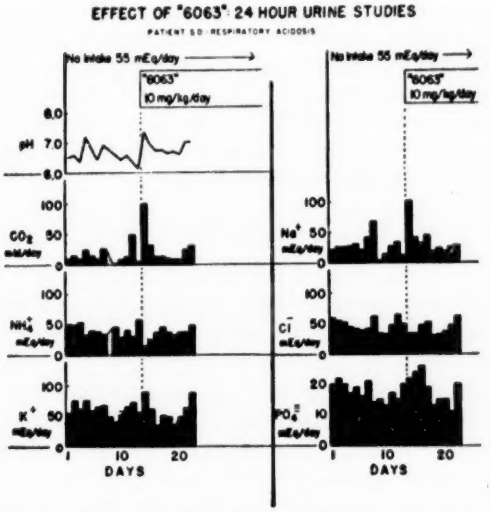

FIG. 5. DAILY 24-HOUR URINE CONSTITUENTS OF PATIENT S. D. (RESPIRATORY ACIDOSIS)

The urine studies were discontinued prematurely because of the development of a urinary tract infection.

"6063" (8 mg. per kg. body weight per day) for three weeks and remained alert. One week after discontinuation of "6063," the patient developed more marked respiratory acidosis with associated drowsiness and neuromuscular irritability. Reinstitution of "6063" therapy (10 mg. per kg. body weight per day) was followed by symptomatic and chemical improvement and, at the time of this writing, (November, 1952), with continued use of the drug, the patient has remained alert without use of

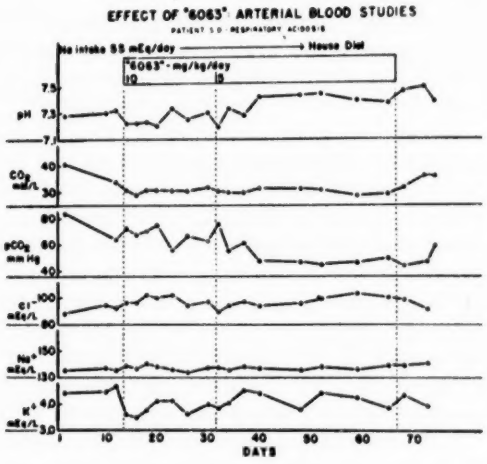

FIG. 6. ARTERIAL BLOOD STUDIES OF PATIENT S. D.

Two weeks after the initiation of "6063" (10 mg. per kg. per day), the patient (S. D.) became drowsy and confused. These symptoms disappeared promptly when the dosage was lowered to 5 mg. per kg. per day.

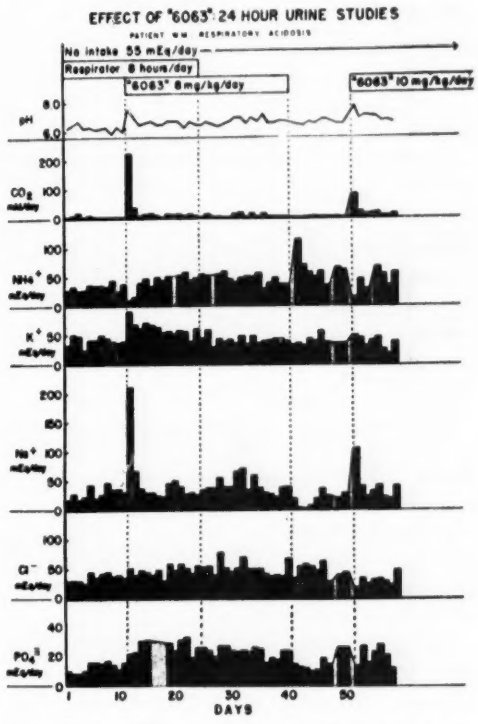

FIG. 7. DAILY 24-HOUR URINE CONSTITUENTS OF PATIENT W. M. (RESPIRATORY ACIDOSIS)

Patient W. M. had been in coma with respiratory acidosis on three occasions in the two months preceding this study. These episodes of coma were not related to changes in oxygen therapy. The coma had cleared on all three occasions following the use of a Drinker respirator, during which time there was a fall in the blood CO_2 content. These studies were started when the patient was still being maintained in a respirator eight hours each day. Two weeks after the initiation of "6063" therapy the patient was removed from the respirator, the "6063" being continued for three additional weeks. The patient remained alert. However, one week after discontinuation of "6063" the patient developed marked drowsiness and neuromuscular irritability and asked to be returned to the respirator. This was associated with a marked rise in arterial blood CO_2 content and a fall in pH. Instead of respirator therapy the patient was given "6063" and his symptoms disappeared promptly.

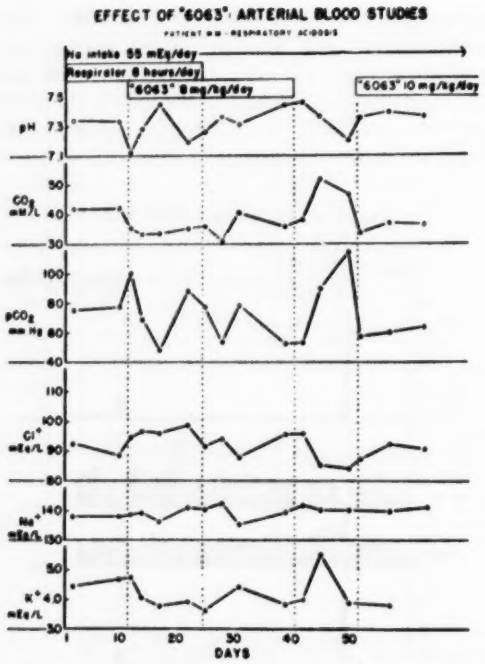

FIG. 8. DAILY ARTERIAL BLOOD STUDIES OF PATIENT W. M. (RESPIRATORY ACIDOSIS)

See legend to Figure 7.

the respirator for three months. No late side effects have been observed.

The other patient (S. D.), with respiratory acidosis but without antecedent alteration of consciousness, developed marked drowsiness 14 days after the initiation of therapy. This was not correlated with changes in the arterial pH or CO_2. These symptoms disappeared promptly when the dose was decreased from 10 to 5 mg. per kg. per day. No other toxic manifestations or clinical changes were noted.

Four additional patients with severe pulmonary emphysema and respiratory acidosis have received oral "6063" in more limited studies. In all four there was an initial fall in the plasma CO_2 content of 5 to 10 mEq. per L. In three patients the CO_2 content remained depressed during the entire two to four week period of "6063" administration. These three patients were alert prior to and during this study. The fourth patient had had drowsiness due to respiratory acidosis. His mental status cleared and his plasma CO_2 content decreased coincident with initiation of "6063" therapy. However, in this latter patient the plasma CO_2 content rose to pre-treatment levels despite continued administration of "6063" and the patient again developed drowsiness which cleared only after five days of Drinker respirator therapy.

DISCUSSION

In normal renal function carbonic acid (H_2CO_3) is believed to be the main source of hydrogen ion necessary for the acidification of the urine, the formation of ammonium ion (NH_4^+) from ammonia (NH_3), and the distal tubular reabsorption of sodium. The reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ ($\rightleftharpoons H^+ + HCO_3^-$) is catalyzed by the enzyme carbonic anhydrase which is found in the renal cortex in high concentration (13). Most of the immediate effects of the administration of an inhibitor of carbonic anhydrase (such as sulfanilamide or "6063") on acid-base equilibrium can be explained by interference with the renal mechanisms in which this enzyme is presumably involved. Inhibition of carbonic anhydrase in the kidney leads to:

1. Decreased acidity of urine due to decreased availability of the hydrogen ion from carbonic acid.

2. Increased urinary excretion of sodium due to disruption of the tubular exchange mechanism (14, 15) in which hydrogen ion is normally excreted by the tubule cell and sodium reabsorbed.

3. Increased urinary excretion of potassium due to increased tubular secretion of potassium. Inhibition of renal carbonic anhydrase is thought to produce a relative alkalosis in the renal tubule cells because of reduction of available hydrogen ion (15, 16). The normally occurring "competition" between hydrogen and potassium in the ion exchange mechanism of sodium reabsorption is disrupted (17, 18). Exchange of potassium for sodium rather than hydrogen for sodium is favored and potassium secretion is increased (15).

4. Decreased ammonium ion (NH_4^+) excretion due to limited availability of hydrogen ion for combination with ammonia (NH_3); the decreased ammonium ion formation also leads to enhanced urinary excretion of sodium (18).

TABLE I

Effect of "6063" on urine constituents of four subjects

Constant diet containing 55 mEq. sodium per day*

Item	Patient	Control period 1			Drug period ("6063") 8–18 mg./kg./day				Withdrawal period	Control period 2		
						Drug period after day 2						
		N	Mean	S.D.	Day 1	N	Mean	S.D.	Mean for 5 days after "6063" discontinued	N	Mean	S.D.
pH	W. B.	9	6.48	0.119	7.26	25	6.45	0.249	6.21 (min. value 5.95)	12	6.63	0.223
	M. W.	12	6.20	0.170	7.38	22	6.54	0.224	6.21 (min. value 6.15)			
	W. M.	11	6.26	0.231	7.60	25	6.65	0.195	6.25 (min. value 6.40)			
	S. D.	11	6.67	0.313	7.37							
CO_2 mM/day	W. B.	10	10.1	3.55	93.6	25	9.7	2.66	5.3 (min. value 1.8)	12	7.5	3.96
	M. W.	12	4.8	2.31	83.4	22	7.5	4.33	1.9 (min. value 1.1)			
	W. M.	11	7.5	4.35	226.0	25	10.9	5.34	4.9 (min. value 3.2)			
	S. D.	12	15.2	11.77	99.3							
NH_4^+ mEq./day	W. B.	10	30.6	4.39	5.6	25	28.6	4.41	55.6 (max. value 76.1)	12	34.9	5.84
	M. W.	13	27.8	4.78	10.9	22	27.2	7.93	57.8 (max. value 67.4)			
	W. M.	11	33.7	5.53	9.5	25	47.5	7.38	73.2 (max. value 113.7)			
	S. D.	12	41.7	9.16	16.0							
Na^+ mEq./day	W. B.	11	44.3	8.46	102.0	27	43.4	9.10	25.3 (min. value 9.9)	14	42.1	22.09
	M. W.	13	29.1	7.59	84.5	24	28.0	9.39	14.3 (min. value 4.2)			
	W. M.	11	29.6	9.09	211.9	27	38.7	12.69	10.8 (min. value 3.2)			
	S. D.	13	28.1	15.23	101.0							
K^+ mEq./day	W. B.	11	72.0	7.75	118.2	27	60.9	8.62	49.4 (min. value 32.4)			
	M. W.	13	54.4	12.03	107.0	24	48.2	14.68	17.0 (min. value 11.2)			
	W. M.	11	40.2	6.97	90.0	27	47.2	10.45	35.7 (min. value 30.6)			
	S. D.	13	61.2	10.31	88.0							
Cl^- mEq./day	W. B.	11	58.0	7.44	43.8	27	52.3	7.38	66.9 (max. value 89.7)	14	51.1	19.26
	M. W.	13	42.4	8.98	33.6	24	34.3	7.29	41.6 (max. value 47.7)			
	W. M.	11	35.4	6.01	51.7	27	49.9	10.62	49.6 (max. value 57.7)			
	S. D.	13	48.1	9.65	32.2							
$PO_4^=$ mEq./day	W. B.	11	22.8	2.03	27.6	24	24.7	2.86	18.5 (min. value 15.4)	14	19.7	1.04
	M. W.	13	17.5	3.71	25.4	21	18.5	3.59	15.0 (min. value 11.7)			
	W. M.	11	12.8	3.25	21.7	24	24.6	4.43	12.3 (min. value 9.7)			
	S. D.	13	17.4	3.00	22.2							

* N—Number of determinations.
S.D.—Standard deviation.

5. Increased total CO_2 content of urine, due mainly to increased excretion of bicarbonate. Normally, hydrogen ion secreted by the renal tubule cells reacts with the bicarbonate in the tubular urine; the CO_2 so evolved diffuses into the blood. When hydrogen ion secretion is diminished by carbonic anhydrase inhibition this reaction does not take place and bicarbonate excretion is thereby increased (18, 19).

The acute loss of large quantities of sodium and potassium bicarbonate *via* the urine is associated with a fall in plasma bicarbonate and blood pH. A significant fall in the plasma potassium concentration occurred in three of the four patients studied. No change in plasma sodium occurred.

Increased urinary excretion of phosphate occurred after the administration of "6063." Increased urinary phosphate occurs also in acidosis from other causes (20). The mechanism is not known.

With prolonged administration of "6063" the changes in urinary excretion of electrolytes lasted only one to two days. Thereafter, urine electrolytes were essentially the same as during the control period. The nature of this apparent urinary compensation is not known. There are several possible explanations. There may be renal mechanisms other than that involving carbonic anhydrase which can make hydrogen ion available; increased amounts of carbonic anhydrase may permit the

normal reaction to proceed in spite of the presence of the inhibitor. All of the reactions may not require an enzymatic catalyst.

With the exception of the CO_2 content, the changes in arterial blood electrolytes tended to return to control values after one to two weeks of continued drug therapy. Although the arterial pH tended to return to normal, the plasma CO_2 content remained depressed (5 to 10 mEq. per L.) during the entire period of administration of "6063" in both control subjects and in five of the six patients with respiratory acidosis studied. This was due to a fall in the arterial pCO_2. As this is completely under pulmonary control, the fall in arterial pCO_2 associated with a rise in arterial pH must be attributed to respiratory compensation for the metabolic acidosis, *i.e.*, hyperventilation. The patients with respiratory acidosis and control CO_2 tensions of about 80 mm. Hg initially must have had either a) pulmonary ventilation impaired to the point that effective diffusion of CO_2 from the blood was impossible despite maximal ventilatory effort, or b) insensitivity of the respirator center and/or chemoreceptors to the stimulus of a high arterial pCO_2. It is interesting to note that the pulmonary dissipation of CO_2 is apparently more effective following the administration of "6063". The mechanism is obscure.

Because carbonic anhydrase is thought to be important in the secretion of acid in the gastric juice (21), gastric analyses were performed before and during "6063" therapy in the control patients. There was no significant change in gastric secretion of free or combined acid or of the electrolytes of the gastric juice before or after histamine stimulation one day or seven days after the initiation of "6063" in the doses used.

"Rebound phenomena" after cessation of "6063" are prominent. They last for more than one week and consist of decreased urinary excretion of sodium and in one case of potassium, increased ammonia and decreased phosphate excretion. There was a rise of blood pH to above control levels, and a return of blood CO_2 content to control levels only after about one week. The retention of sodium after withdrawal of "6063" indicates that the initial large loss of sodium, on administration of the drug, may not be made up while "6063" is administered even though urinary output of sodium is the same as during the control period. One patient (M. W.) was given 170 mEq. of sodium per day as NaCl in capsule form, in addition to a diet containing approximately 50 mEq. of sodium per day. After a control period of one week "6063" was given as previously described. The effects of the drug were the same as with the smaller sodium intake except for the baseline levels of the control period. Withdrawal of "6063" was followed by a period of diminished sodium excretion from control levels of about 150 mEq. per day to about 20 mEq. per day for several days. Thus, "rebound phenomena" were present in spite of a relatively large continuous intake of sodium. The explanation for these "rebound phenomena" is obscure. It is possible that the same mechanism that caused urinary excretion of electrolytes to return to control levels during prolonged administration of "6063" continued to operate for some time after the drug was discontinued.

SUMMARY

1. The effects of administration of the carbonic anhydrase inhibitor "6063" on electrolyte and acid-base metabolism have been studied in two normal subjects and two patients with pulmonary emphysema and respiratory acidosis. The electrolyte changes in both groups were essentially the same.

2. Administration of "6063" causes transient increased urinary pH, increased urinary CO_2, sodium, potassium and phosphate and a fall in urinary ammonia. Associated with this there is a fall in arterial pH and CO_2 content. There is a transient decrease in the plasma potassium concentration. After two to three days the daily urinary excretion of electrolytes returns to the levels of the control period, but the plasma CO_2 content remains depressed during the entire period of drug administration and the arterial pH only gradually rises to the control levels. Upon cessation of "6063" therapy, there is decreased urinary excretion of sodium, increased urinary ammonia and a rise of blood pH to above control values for three to seven days.

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STUDIES ON THE PREVENTION OF RHEUMATIC FEVER: THE EFFECT OF TIME OF INITIATION OF TREATMENT OF STREPTOCOCCAL INFECTIONS ON THE IMMUNE RESPONSE OF THE HOST [1]

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(Submitted for publication February 23, 1953; accepted March 18, 1953)

Previous studies have established the fact that penicillin treatment of acute streptococcal pharyngitis results in a marked suppression of antibody formation (1, 2, 3). This is in contrast to the failure of penicillin treatment to alter the immune response in patients with pneumococcal pneumonia (4). It has been suggested that in the latter case, sufficient pneumococcal antigen is formed during the early phases of pneumonia to provide a maximal antigenic challenge before penicillin therapy is instituted (4). The capacity of penicillin to suppress the production of streptococcal antibodies suggests that in streptococcal infections the antigenic challenge may be presented more gradually. Under such circumstances, it would be expected that the effectiveness of penicillin in this regard would be related to the time of institution of therapy. Since no definitive information on this point has hitherto been obtained, the present study was designed to test this hypothesis.

DESCRIPTION OF STUDY

All patients fulfilling the following criteria were included in the study: the presence of exudative pharyngitis, history of onset of symptoms less than 31 hours before admission, and subsequent isolation of group A streptococci from the initial throat culture. Patients with a past history of rheumatic fever or chorea were excluded. A throat culture and a serum specimen were obtained on each patient at the time of admission and on the fourteenth, twenty-first and twenty-eighth days from the onset of symptoms.

Bacteriological and serological studies were performed by methods previously described (5, 6, 7). Antistreptolysin "O" titers were determined on all sera from each patient in a single test, and all tests were performed with the same lot of streptolysin and read by the same observer.

RESULTS

Each of the 349 patients studied was assigned to one of four treatment groups according to a random sequence previously determined by a shuffled deck of cards. Table I indicates the distribution of the patients among four treatment groups according to the time of initiation of therapy and the amounts of penicillin employed. The groups were essentially comparable, as shown in Table II, with respect to the admission leukocyte count, predominance of group A streptococci on initial throat culture, history of sore throat within the previous four weeks, and initial antistreptolysin titer. There was some variation in the distribution of serological types of infecting streptococci in the four treatment groups, and analysis revealed only slight differences in the antistreptolysin responses to infection with the different types.

Table III shows the average increase in antibody in each group. Early treatment with penicillin was followed by a much smaller average antibody rise than that found in the control group. In those groups of patients in which treatment was delayed for three or five days, there was progressively less suppression of antibody response. Nevertheless, considerable reduction in antibody response was obtained even when treatment was delayed for five days.

Figure 1 shows the per cent of patients in each group who developed an antibody rise of 200 units or over. It can be seen that twice as many patients developed this response when treatment was de-

[1] This investigation was conducted under the sponsorship of the Commission on Acute Respiratory Diseases and the Commission on Streptococcal Diseases, Armed Forces Epidemiological Board, and was supported by the Offices of The Surgeons General, Departments of the Army and Air Force, Washington, D.C.

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TABLE I

Distribution of cases according to treatment groups

Group	Number of cases	Time of initial injection*	Therapy
Immediate treatment	86	22–31 hours	Three injections of 600,000 units of penicillin† at 3 day intervals
3 day delay in treatment	89	70–79 hours	
5 day delay in treatment	87	118–127 hours	
Control	87	—	Saline placebos given on first and fifth hospital day

* Time from onset of symptoms.
† Procaine penicillin G in sesame oil, with 2 per cent aluminum monostearate.

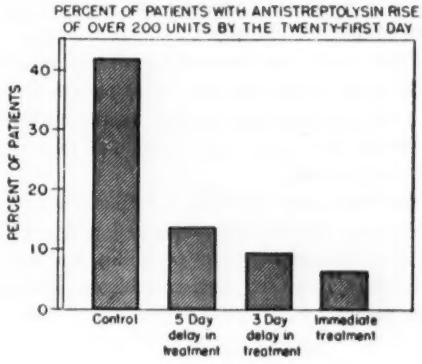

FIG. 1

TABLE II

Comparability of the treatment groups

		Number of patients in treatment groups			
		Immediate treatment (86)	*3 day delay in treatment (89)*	*5 day delay in treatment (87)*	*Control (87)*
Admission leucocyte count 12,000 and over		64	63	65	69
Initial antistreptolysin titer 125 units and under		56	66	64	65
History of sore throat within previous 4 weeks		8	12	7	11
Group A streptococci predominant* growth on initial culture		81	79	76	81
Type of group A streptococci from initial throat culture	14	10	14	20	20
	30	19	26	23	28
	3	9	6	4	2
	18	15	14	13	13
	6	8	9	8	6
	5	10	9	10	7
	Others	15	11	9	11

* Over 50 colonies on plate.

TABLE III

Average antistreptolysin response in relation to time of initiation of penicillin therapy

Treatment groups	Number of cases	Antistreptolysin "O" titers									
		Average units				Average units rise			Per cent inhibition as compared to controls		
		Initial	*14 day*	*21 day*	*28 day*	*14 day*	*21 day*	*28 day*	*14 day*	*21 day*	*28 day*
Immediate treatment	86	137	214	224	231	77	87	94	58.4	60.3	56.0
3 day delay in treatment	89	121	205	229	235	84	108	114	55.0	50.3	46.8
5 day delay in treatment	87	120	222	251	246	103	131	126	44.8	40.0	41.1
Control	87	119	304	337	333	186	218	214	—	—	—

layed five days, compared to the group who received immediate therapy.

In this study one case of rheumatic fever occurred in the control group. This patient showed a 375 unit antistreptolysin rise by the fourteenth and twenty-first days, and a 750 unit rise by the twenty-eighth day.

Reinfections and recurrences of positive cultures at follow-up examination remained under 13 per cent in all groups receiving penicillin, and did not appreciably alter the antistreptolysin responses of any group.

DISCUSSION

These data indicate that there is a correlation between the time of institution of penicillin therapy and the degree of suppression of antistreptolysin formation: the earlier the treatment the greater the suppression. Therapy, begun not later than 31 hours after the onset of symptoms of streptococcal pharyngitis, effectively reduces the subsequent antistreptolysin response at 21 days by approximately 60 per cent, while therapy begun five days after the onset of symptoms results in a 40 per cent suppression. These observations support the hypothesis that the antigenic challenge during acute streptococcal pharyngitis occurs gradually, over a period of several days, and that an appreciable percentage of the total immune response develops as a result of antigen formed after the fifth day of disease.

It is difficult to relate these data to the overall problem of the prevention of rheumatic fever, since the present study was too limited to yield any direct evidence as to the effectiveness of delayed penicillin therapy in reducing the attack rate for this complication. It has been shown that there is a correlation between high convalescent antibody titers following streptococcal infections and the development of rheumatic fever (8). It would thus seem that early therapy of streptococcal infections would be advisable; delayed therapy, however, might exert some effect on the incidence of rheumatic fever. It is hoped that studies now in progress will provide information on this point.

CONCLUSIONS

Early treatment of streptococcal pharyngitis with penicillin is more effective than delayed therapy in suppression of antistreptolysin formation. Treatment instituted as late as five days from onset of symptoms of pharyngitis results in significant antibody suppression.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Captain Harold I. Griffeath and Captain Robert S. Fontana of the medical service for their assistance and cooperation.

Members of the staff of the Streptococcal Disease Laboratory who assisted in these studies include Dr. Charles H. Rammelkamp, Director, Captain Lewis W. Wannamaker and Captain Chandler A. Stetson, Assistant Directors, and Major Edward O. Hahn, Major Harold B. Houser, Captain William D. Perry, Captain Bertrand L. Stolzer, and T/Sgt William H. Stroud.

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DEPRESSOR EFFECTS OF POTASSIUM-DEFICIENT DIETS IN HYPERTENSIVE MAN [1]

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(Submitted for publication March 6, 1953; accepted April 1, 1953)

The relationship of the sodium ion to blood pressure levels in certain hypertensive states is now established beyond cavil. But whether alterations are mediated through the adrenal cortex, through renal or other pressor mechanisms, or through modification of neuromuscular responses at an arteriolar plane, cannot be stated at the present time.

In recent years the potassium ion has been considered in other ways than its known effects on cardiac muscle contraction or on synaptic transmission. Potassium chloride-flooding in rats has been reported to induce an increased renal and a decreased cardiac weight as well as hypertrophy of the adrenal glomerulosa zone (1). Furthermore, Freed, Friedman and Rosenman have demonstrated that dietary potassium restriction will lower the blood pressure in normal and hypertensive rats, block the pressor effects of desoxycorticosterone, and reduce peripheral vascular reactivity (2–5).

The present study was undertaken to investigate the effects of potassium-deficient diets in hypertensive man, with the hope that further light might be thrown on the mechanisms involved in sodium restriction.

METHODS

Five observations were made on four patients with hypertensive vascular disease, uncomplicated save that two had sustained relatively minor cerebral vascular accidents at least a year before. The conditions, diagnostic procedures and methods [2] were similar to those reported previously (6), including the utilization of "resting" blood pressure measurements, constant dietary and fluid intakes, and preliminary periods of hospital supervision of about three weeks. Desoxycorticosterone acetate was administered subcutaneously in 5 mg. doses once daily.

Identical salt-poor daily menus were prepared as before (6), with sodium chloride administered by mouth from weighed salt shakers to yield a total daily intake of about 4 grams. Potassium restriction was achieved (without change in total fluids, calories, sodium or the addition of special ingredients) by almost complete elimination of fruit juices, potato, milk and eggs, reduction of meat and coffee, selection of certain fruits, cereals and vegetables, and the addition of fats, sugar and ginger ale. This resulted in a decreased protein intake (ranging in different patients from 69 to 84 grams per day in the control period, to 31 to 48 grams while on the curtailed regimen) and an increase in the consumption of fat. Repeated direct analyses of aliquots taken from an entire day's cooked diet confirmed the constancy of the regimen.

RESULTS

Tabular representation of the results is summarized in Table I and an illustrative case record is shown in Figure 1. The restriction of potassium, to approximately a third or a quarter of normal intake, was associated with a small but consistent and statistically significant decrease in

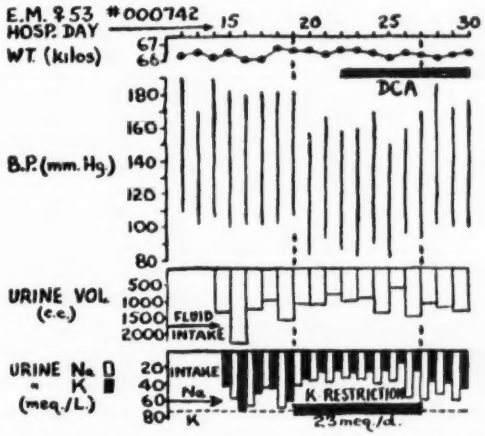

FIG. 1. CLINICAL AND LABORATORY DATA OF HYPERTENSIVE PATIENT GIVEN POTASSIUM-DEFICIENT DIET

[1] This study was supported by grants from the Albert and Mary Lasker Foundation, the Albert H. and Jessie D. Wiggin Foundation, and the National Heart Institute (USPHS).

[2] The author is indebted to Dr. James A. L. Mathers for ballistocardiographic studies with the low frequency, critically-damped instrument, and to Dr. K. W. Thompson of Organon, Inc., Nutley, N. J. for supplying the desoxycorticosterone acetate (DOCA).

TABLE I

Effects of potassium-deficient diets in hypertensive subjects

	Diet; duration *days*	K intake *mEq./d.*	"Resting" B.P. Max./Min.	"Resting" B.P. Mean	Urine (av./day) Vol. *cc.*	Urine (av./day) Na *mEq.*	Urine (av./day) K *mEq.*	Serum Na *mEq.*	Serum K *mEq.*	Serum Cl *mEq.*	Serum CO_2 *mEq.*	H'crit *% cells*	Weight Start–End *kilos*
P. I.	control (10)	94	176/112 – 166/106	170/108	2215	67	75	138	4.4	102	30.1	47	
	low K (9)	23	172/102 – 154/92	164/100	1998	41	52	141	4.1	102	31.5	46	74.4–74.0
E. M.	control (8)	72	190/110 – 170/100	182/105	1472	60	58	137	4.1	102	27.1	36	66.4–66.6
	low K (3)	23	166/92 – 158/84	161/87	1277	34	26	137	4.3	104	28.1	34	66.6–66.6
	low K + DCA (5)	23	170/96 – 150/82	162/89	1060	39	21	137	3.6	105	27.6	36	66.6–66.5
	control + DCA (3)	72	186/102 – 172/100	178/101	1147	57	39						66.5–66.5
L. P. (1)	control (5)	67	180/100 – 170/96	174/99	1810	66	56	142	4.6	102	30.6		67.4–67.0
	low K (3)	21	166/88 – 164/86	165/87	1743	27	31	142	4.4				67.0–66.9
	low K + DCA (4)	21	174/94 – 154/86	162/88	1825	32	30	143	4.0	100	30.6		66.9–67.9
	control + DCA (4)	67	178/100 – 164/90	170/96	1770	51	42						67.9–68
L. P. (2)	control (5)	67	158/90 – 150/86	153/88	1973	65	57	143	4.4	102	30.0		67.1–66.9
	low K (4)	21	146/86 – 142/80	143/82	1900	31	32	142	4.2	100	28.0		66.9–66.9
	low K + 57 mEq. KCl (3)	78	164/96 – 160/90	162/93	2113	91	43						66.9–66.2
	low K (3)	21	158/86 – 154/82	156/84	2166	69	30						66.2–65.9
M. L.	control (10)	80	196/120 – 182/114	189/118	1570	71	63					45	
	low K (8)	20	176/112 – 156/96	164/103	1500	36	38					44	
	control (10)	80	192/116 – 180/110	185/114	1510	68	58					44	

"resting" blood pressure in every instance. This response appeared not later than 48 hours after diet modification and often within 24 hours, and the "resting" blood pressure rose promptly in those cases in which potassium chloride was added to the diet or when control diets were restored.

Curtailment of dietary potassium was followed by a decrease in the amount excreted in the urine, but in general these values remained larger than the intake with a resultant negative balance, perhaps reflected in the serum potassium concentrations which fell slightly in three of four studies. Urinary nitrogen determinations were made in one patient and showed a decrease in proportion to the

reduced protein intake, thereby indicating that protein breakdown was contributing a negligible amount to the urinary potassium excreted during the limited period of these observations. In association with the restriction of potassium, urinary sodium excretion decreased sharply with an average positive balance, assuming other excretory routes were not invoked, of approximately 30 mEq. per day. Serum sodium concentrations, however, were not affected.

Except for a slight increase in constipation in two subjects, the restriction of potassium gave rise to no subjective distress. The shifts in electrolytes were unassociated with any major fluid alterations, at least as reflected in weight, hematocrit and urinary volume measurements, and in one subject the serum volume (calculated by means of the blue dye T. 1824) remained unaffected. Serum chloride and carbon dioxide content were not modified consistently. No patient was observed to develop urinary ketone bodies, and serum urea nitrogen values did not increase. Serial electrocardiograms and ballistocardiograms were obtained at frequent intervals in the course of two studies; the results were superimposable, nothing being found to suggest changes in cardiac function, intracardiac electrolyte alterations, or difference in the impact of the cardiac contraction.

In two instances, desoxycorticosterone acetate was administered while the patients were on the restricted diet. The "resting" blood pressure remained reduced until the control diet was resumed.

DISCUSSION

The decrease in "resting" blood pressure in response to short-term restriction of dietary potassium, observed six times in five studies on four patients with hypertensive vascular disease, confirms the reports of Freed, Friedman and Rosenman employing even greater restriction in hypertensive rats. It is noteworthy, with respect to the known therapeutic effects of salt restriction, that this phenomenon takes place despite rather marked sodium retention and, presumably, a rise in intracellular sodium (7–10).

The mechanism of the fall in blood pressure remains obscure. It appeared unrelated to serum volume changes in the one individual in whom measurements were made. It could not be accounted for by the decrease in protein intake, necessary to achieve the potassium restriction, for the initial degree of hypertension could be restored in one instance merely by the addition of potassium chloride; nor, incidentally, will protein supplementation alter the arterial tension when reduced by the "rice diet" (11). It is doubtful that the depressor effects can be related to loss of potassium from heart muscle and a resultant fall in cardiac output, although this possibility cannot be excluded completely; against such a mechanism are the rapidity of the blood pressure decrease, the absence of abnormally low serum potassium values, and the lack of alterations in the electro- and ballistocardiograms. With reference to the role of the adrenal cortex, one might presume that a high rather than a low potassium intake should be analogous to a low sodium regimen; nevertheless, the fact that a low potassium diet blocks the customary pressor response of hypertensives to desoxycorticosterone (12), also noted previously in the rat studies (4), makes it possible that the adrenal cortex is one of the areas requiring further consideration. Finally, it is possible that electrolyte imbalance exerts a direct effect on neuromuscular transmission and hence a direct effect on the physiological site of hypertension, the arterioles; marked sodium deficiency has been reported to give rise to a neuromuscular block and a reduced output of acetylcholine (13).

From these preliminary studies it cannot be concluded that potassium restriction is of therapeutic benefit. The diets employed were unpalatable and unbalanced, particularly with respect to protein. Had they been made more restrictive or continued for longer periods, signs and symptoms and dangerous cardiac complications might have been produced. These observations do serve, however, to point again to the relationship of certain cations to the hypertensive state—perhaps through the intervention of the adrenal cortex, but perhaps through some direct action on blood vessel walls.

SUMMARY

1. The restriction of dietary potassium, undertaken six times in four patients with hypertensive vascular disease, was followed rapidly by small but statistically significant decreases in "resting" blood pressure.

2. Sodium retention accompanied the restriction of potassium.

3. The low potassium diet blocked the pressor response to desoxycorticosterone.

4. This study, confirming previous reports of hypotension observed in potassium-deficient rats, fails to exclude the possible participation of the adrenal cortex but is compatible also with the possibility of a direct role of cations upon neuromuscular transmission or upon blood vessel walls during the hypertensive state.

ACKNOWLEDGMENTS

The author acknowledges gratefully the assistance of Margaret Crymble, Iva Moen, Mary Iles and Margaret N. Hawthorne.

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THE CHARACTERISTICS OF THORACIC DUCT LYMPH IN MAN

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(Submitted for publication January 17, 1953; accepted April 4, 1953)

The thoracic duct is generally accepted as the major pathway of lymphocytes enroute to the circulating blood (1, 2, 3), accounting for approximately 70 per cent of all the lymphocytes in the peripheral blood. The remainder of the lymphocytes are said to enter the blood directly via the lymph from other sites (2). Reliable information concerning the hematologic content of lymph in the thoracic duct of man has been fragmentary and limited to chance observations in few patients (4).

The thoracic duct lymph is reported to flow from one to six hours after death (5, 6). Consequently, an attempt was made to study the cellular components of human lymph obtained by cannulation of the thoracic duct within one hour after death and then *in vivo* if a feasible technic could be developed (7). Volumetric measurements of the flow of lymph in addition would permit an estimation of the number of cells delivered per unit time into the peripheral circulation via the thoracic duct (8). Since at least two thoracic ducts are present in man in addition to innumerable other lymph-to-blood connections, it was realized that these anatomical considerations seriously limited the interpretation of single samples. Consequently cannulation of the major thoracic duct for continuous drainage over prolonged periods was undertaken.

PATIENTS AND METHODS

The thoracic ducts of 16 cadavers were isolated within one hour after death. Satisfactory flow of lymph fluid was obtained in 11 of these necropsy cases, 10 of whom had a form of leukemia. The hematologic findings were contrary to that expected from a review of the literature. Because of a valid criticism of any interpretation derived from *post mortem* material, the study was extended to living man (Figure 1).

Studies were performed on 10 patients, all of whom were far advanced in the course of their neoplastic disease, and all of whom volunteered as subjects after being informed of the proposed study. Four patients had leukemia, and six had other malignancies. Four ounces of milk and cream were given orally one-half hour prior to the procedure to aid in the identification of the duct. Polythene tubing, 2 to 3 mm. in external diameter, was introduced 3 to 5 cm. into the proximal segment of the thoracic duct pointing retrograde as it approached the left internal jugular-subclavian vein junction. The segment of the duct emptying into the venous junction was ligated. The polythene tubing pointing medially and inferiorly was securely tied in place after flow was established. The skin was closed with interrupted sutures without approximating the deeper layers. The tubing was fixed by sutures within the edges of the wound as it was closed.

The lymph was collected by gravity drip into sterile, silicone-lined glass containers. Heparin was employed exclusively as the anticoagulant. Samples for hematologic or chemical determinations were collected as separate fresh specimens. All counts and determinations were done as promptly as possible employing NBS certified hemocytometers and Trenner automatic filling pipets. In counts between 5,000 to 10,000 per cu. ml., counting all nine squares on each of two chambers from a single pipet, the 70 per cent confidence limits of the value obtained were ± 8.5 per cent. Peripheral venous blood was taken simultaneously for comparison. In the patients studied *post mortem*, blood for comparison had been obtained either just before death or by cardiac puncture after death.

In five instances, the thoracic duct was outlined *in vivo* by the retrograde introduction of Diodrast or Thorotrast (Figures 2A, B, C, D).

Whole blood or plasma was employed to replace the draining lymph, usually volume for volume. After the period of the lymph drainage was completed, the tubing was sealed and the lymph within was allowed to clot. The tube was uneventfully withdrawn 3 to 10 days later.

RESULTS

Studies post mortem. Attempts to locate the thoracic duct *post mortem* were uniformly unsuccessful unless half cream-half milk had been ingested 3 to 24 hours before cannulation. Satisfactory lymph flow was obtained in 11 patients, 10

of whom had leukemia (Table I). In eight patients, the blood leukocyte level exceeded the leukocyte level of the thoracic duct lymph; in two patients the reverse was found to be true. The leukocyte counts were the same in the lymph and blood of the remaining patient.

Studies in vivo. The thoracic duct was clearly distinguishable *in vivo* when the cream and milk were given 15 to 30 minutes before surgical exploration of the duct area. Thoracic duct lymph was obtained for continuous periods of 2 to 13 days in 10 patients (Table II, Figure 3). The polythene tube caused no untoward reaction during or after the period of drainage and did not seriously impair motion of the head or neck or cause discomfort after the first 24 or 48 hours. Delayed healing of the wound occurred in an area which had been repeatedly treated with intensive X-radiation in a single patient (KIN) who had advanced Hodgkin's disease. No evidence of a chylous fistula appeared in any case. One patient experienced transient syncope during the introduction of 70 per cent Diodrast into the thoracic duct although intravenous testing had shown no sensitivity.

FIG. 1. CANNULATION OF THE THORACIC DUCT
The lymph is collected in sterile containers by gravity drip.

Appearance and flow characteristics. The lymph of the non-leukemic and leukemic patients had an opalescent appearance resembling skimmed milk, varying between a thick creamy and a thin watery consistency. Cream appeared within 10 to 20

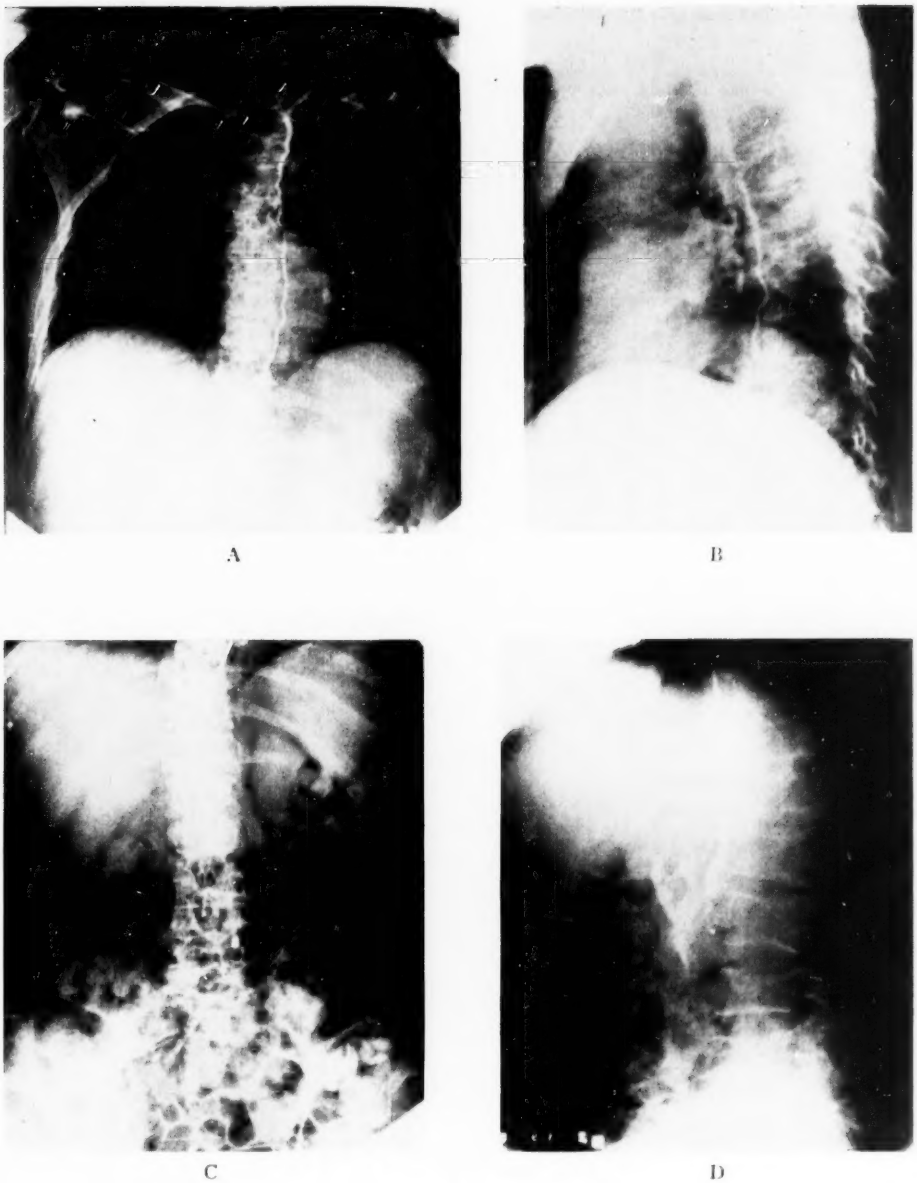

FIG. 2

A. Outline of thoracic duct in thorax following retrograde introduction of 75 per cent Diodrast.

B. Lateral view.

C. Anterior view of abdominal portion of the thoracic duct. The marker "O" is at the umbilicus.

D. Lateral view of abdominal portion. Note filling anteriorly of what is presumed to be mesenteric lymphatics.

TABLE I

Hematological data of thoracic duct lymph of eleven patients obtained at post mortem compared with venous blood

Name, Sex, Age Diagnosis	LIB F 3 Lymphatic leukemia		LEE F 11 Lymphatic leukemia		CUM M 11 Lymphatic leukemia		DEM M 19 Lymphatic leukemia		KAN F 35 Lymphatic leukemia		JOH M 51 Lymphatic leukemia		
	Lymph	Blood	Lymph	Blood	Lymph	Blood	Lymph	Blood	Lymph	Blood	Lymph	Blood	
Sample site	Thoracic duct	Finger, 1 hr.*	Thoracic duct	Finger, 1 hr.*	Thoracic duct	Cardiac	Thoracic duct	Cardiac	Thoracic duct	Cardiac	Thoracic duct	Vein, 5 hrs.*	Pericardial fluid
RBC	650,000		460,000	Hbg., 5.8 gms.	580,000	1,900,000	100,000	1,250,000	400,000	1,160,000	770,000	2,680,000	30,000
Platelets			80,000					130,000	30,000	40,000		60,000	
WBC	50,200	50,000	241,000	322,000	100	800	6,550	330,000	82,000	500,000	3,600	800	1,900
Differentials in per cent													
PMN		1				4 seen			6	5		76	
Meta										2			
Myl								2		3			
Lymphs, small	80	99	80	80	20 seen	16 seen	94	13	80	32	100	22	
large	20		20	20			6	85	58	58			
Others					1 normoblast	1 normoblast						2 monocytes	

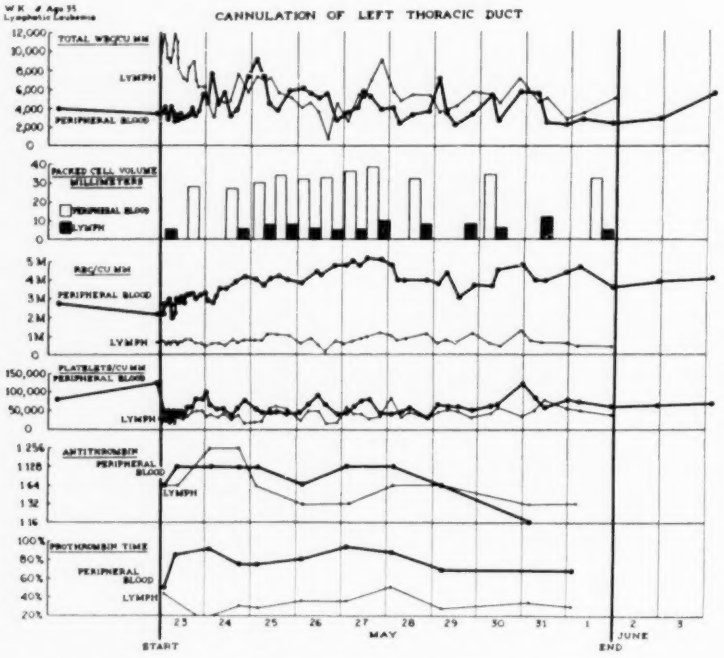

FIG. 3. HEMATOLOGICAL DATA DURING A REPRESENTATIVE STUDY ON KIR, 35-YEAR-OLD MAN WITH SUBLEUKEMIC LYMPHATIC LEUKEMIA

Note that the blood and lymph leukocyte counts approximate one another closely except for the first 24 hours.

TABLE I—*Continued*

Name, Sex, Age Diagnosis	PIT M 66 Lymphatic leukemia		VAL F 68 Lymphatic leukemia		SPI M 47 Myelogenous leukemia		COO M 49 Myelogenous leukemia		LIN M 58 Malignant melanoma	
	Lymph	Blood	Lymph	Blood	Lymph	Blood	Lymph	Blood	Lymph	Blood
Sample site	Thoracic duct	Ear, 5 hrs.*	Thoracic duct	Finger, 1 hr.*	Thoracic duct	Cardiac	Thoracic duct	Ear, 2 days*	Thoracic duct	Ear, 4 hrs.*
RBC	240,000	Hbg., 8.0 gms.	350,000	3,260,000	280,000	1,580,000	740,000	Hbg., 11.8 gms.	36,000	2,130,000
Platelets		80,000	10,000	55,000	10,000	10,000				100,000
WBC	55,000	289,000	35,600	381,000	3,000†	403,000	80,000	18,450	2,700	10,500
Differentials in per cent										
PMN			2		70	31	2	13	5‡	86
Meta				2				31		2
Myl			1	1		68	38	16		1
Lymphs, small	100	100	93	95	30	1	60	34	3	11
large			4	2					2	
Others								PMB, 3 PME, 3		

* Before death.
† One cc. sample obtained diluted by 0.2 cc. of heparin. Counts were not corrected for this dilution.
‡ Ten cells were differentiated. The remainder of the leukocytes showed degenerative changes. The erythrocytes appeared unaltered.

minutes after oral ingestion. In the leukemic patients, the lymph was more often blood-tinged, related to its content of erythrocytes (Table II).

The flow of lymph varied from 300 to 2,800 ml. of fluid per 24 hours (Figure 4). Free flow of lymph was obtained and measured accurately in six patients in whom the lymph content of leukocytes was also determined, thereby permitting an estimation of the number of cells passing through the thoracic duct ostensibly for delivery into the peripheral circulation (Table III). Cannulation of the right thoracic duct in patient ELM 36 hours

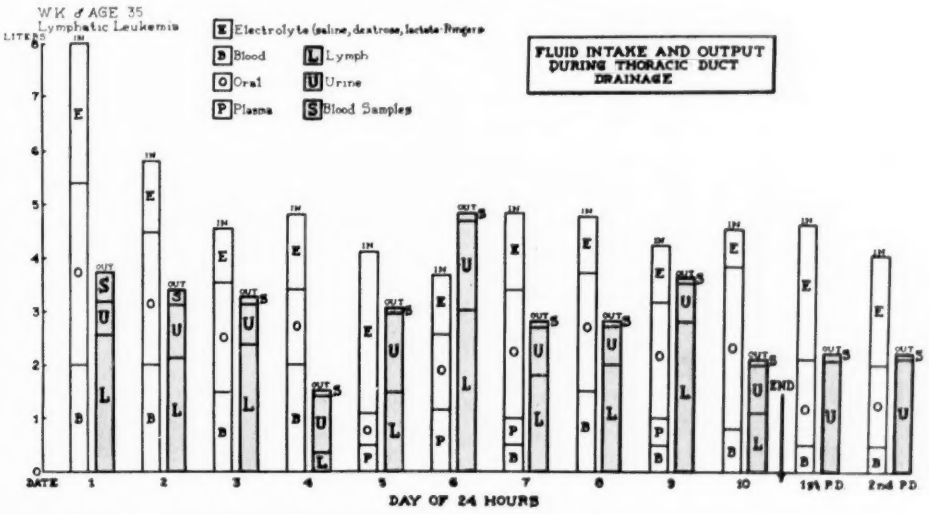

FIG. 4. DATA ON LYMPH FLOW AND FLUID REPLACEMENT DURING STUDY ON PATIENT KIR
The fear of significant fluid loss failed to materialize under this regimen of replacement.

TABLE II

Hematological data of thoracic duct lymph and venous blood on 10 patients obtained in vivo

Name, Sex, Age	ELM M 71		FLU F 75		KIR M 35		RAL M 16		HAL M 49	
Diagnosis	Lymphatic leukemia		Lymphatic leukemia		Subleukemic lymphatic leukemia		Myeloblastic leukemia with chloroma		Multiple myeloma	
Drainage period	2 days		3 days		11 days		4 days		13 days	
	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood
RBC	156,000	2,690,000	99,000	4,070,000	790,000	2,280,000	450,000	2,390,000	35,000	3,950,000
Platelets	4,500	25,000	4,500	95,000	25,000	25,000	15,000	130,000	95,000	125,000
WBC	6,200	49,000	40,600	166,000	11,900	2,800	27,200	67,000	3,300	2,600
Differentials in per cent:										
PMN				11		11	1	12	1	67
Meta								2		
Myl								44		
PME				5	1	1				5
Lymphs:										
small	100	100	100	83	99	85	87	22	98	16
young							11	20		
Mono						3	1			11
Others				1					Plasma cell, 1	PMB, 1
	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood
RBC	174,000	2,380,000	6,300	3,000,000	150,000	4,390,000	100,000	2,080,000	5,000	4,030,000
Platelets	2,500	25,000	4,000	90,000	5,000	60,000	20,000	110,000	5,000	85,000
WBC	3,900	39,000	17,800	121,000	600	4,200	10,000	42,100	210	1,900
Differentials in per cent:										
PMN				2	1	16		10		64
Meta							1	2		
Myl		1						2		
PME								1		
Lymphs:										
small	100	99	100	98	99	84	93	38	4 seen	28
young							6	25		
Mono								2		8
Others										
	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count
RBC	156,000	2,690,000	99,000	4,070,000	895,000	3,720,000	450,000	2,390,000	35,000	3,490,000
Platelets	4,500	25,000	4,500	95,000	25,000	45,000	15,000	130,000	10,000	95,000
WBC	6,200	49,000	40,600	166,000	6,900	8,500	27,200	67,000	1,200	8,300
Differentials in per cent:										
PMN				11	1	13	1	12		80
Meta								2		
Myl								44		
PME				5						3
Lymphs:										
small	100	100	100	83	99	87	87	22	100	5
young							11	20		
Mono				1			1			10
Others										PMB, 1
										Plasma cell, 1
	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count
RBC	132,500	2,630,000	6,300	3,000,000	7,900,000	4,400,000	245,000	3,110,000	5,000	4,030,000
Platelets	2,000	25,000	4,000	90,000	55,000	75,000	45,000	75,000	5,000	85,000
WBC	4,000	25,000	17,000	121,000	2,500	2,100	22,700	23,800	210	1,900
Differentials in per cent:										
PMN				2	4	20		28		
Meta	1							3		
Myl								3		
PME						1				
Lymphs:										
small	99	100	100	98	96	79	98	21	4 seen	
young							2	44		
Mono								1		
Others										

after the left thoracic duct had been ligated did not significantly alter the circulating lymphocyte count.

Cellular characteristics. In general the leukocyte counts in lymph and venous blood in the living patient were the same as found in the *post mortem* studies. The peripheral blood leukocyte count in one patient with chronic lymphocytic leukemia (FLU) varied from 121,000 to 166,000 per cmm., and the comparable leukocyte counts in the lymph were 17,800 to 40,600 per cmm. In another pa-

TABLE II—*Continued*

Name, Sex, Age	CLI F 56		KIN M 16		BUT M 54		BEA M 62		DAV F 28	
Diagnosis	Multiple myeloma		Hodgkin's disease		Carcinoma mouth		Carcinoma neck		Lymphosarcoma	
Drainage period	24 hours		5 days		7 days		3 days		10 days	
	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood	Highest lymph count	Corresponding blood
RBC	1,000	2,670,000	70,000	3,580,000	7,500	4,330,000	2,000	3,740,000	5,000	6,400,000
Platelets	5,000	60,000	50,000	170,000	10,500	295,000	2,000	215,000		
WBC	2,900	1,800	400	6,500	3,500	5,600	5,600	7,900	900	7,600
Differentials in per cent:										
PMN		59	9 seen	94		95		88		86
Meta										
Myl										
PME						1		2		5
Lymphs:										
small	100	30	1 seen	6	100	2	100	10	100	9
young										
Mono		11				2				
Others										
	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood	Lowest lymph count	Corresponding blood
RBC	700	2,220,000	24,500	3,080,000			5,000	3,980,000	few	4,480,000
Platelets	2,000	90,000	8,000	130,000			5,500	210,000		
WBC	1,100	2,400	11	3,600	1,600	4,800	4,000	7,900	None seen	7,000
Differentials in per cent:										
PMN		46		87		83		76		83
Meta										
Myl										
PME				1		1				7.5
Lymphs:										
small	100	50	1 seen	2	100	9	100	20		5
young										
Mono		2		10		7		4		3.5
Others		Plasma cells, 2								PMB, 1
	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count	Corresponding lymph	Highest blood count
RBC	700	2,220,000	70,000	3,580,000	8,000	4,760,000	5,000	3,980,000	5,000	6,400,000
Platelets	200	90,000	50,000	170,000	1,000	200,000	5,500	210,000		
WBC	1,100	2,400	400	6,500	3,000	10,300	4,000	7,900	900	7,600
Differentials in per cent:										
PMN		46	9 seen	94		94		76		86
Meta										
Myl										
PME										
Lymphs:										
small	100	50	1 seen	6	100	2	100	20	100	9
young										
Mono		2				4		4		
Others		Plasma cells, 2								
	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count	Corresponding lymph	Lowest blood count
RBC	1,000	2,670,000	11,500	3,790,000			6,000	3,240,000	few	4,480,000
Platelets	5,000	60,000	4,000	170,000			1,500	205,000		
WBC	2,900	1,800	150	3,200	1,600	4,800	4,500	5,800	None seen	7,000
Differentials in per cent:										
PMN		59	1 seen	69		83		84		83
Meta										
Myl										
PME				1		1		1		7.5
Lymphs:										
small	100	30	1 seen	6	100	9	100	12		5
young			1 seen							
Mono		11		24		7		3		3.5
Others										PMB, 1

tient who had the same disease (ELM) the peripheral blood leukocyte number fluctuated between 25,000 and 49,000 per cmm., while that in the lymph was found to be 4,200 to 6,200 per cmm. The differential leukocyte counts showed smaller total numbers of lymphocytes in the lymph than in the blood in both cases (Table II).

On two occasions, the leukocyte level of the lymph initially exceeded that found in the venous blood. In one patient (KIR) with subleukemic lymphocytic leukemia, except for the first 24 hours, the leukocyte contents of the lymph and blood both varied considerably but in general were approximately the same (Figure 3). Similarly, in another

TABLE III

Calculated total leukocyte flow from the thoracic duct in six patients compared to leukocyte number in the peripheral blood

Patient	Diagnosis	Period of flow	Flow per 24 hours ml.	Average leukocyte count per cmm.		Total leukocyte flow per 24 hours in the lymph	Body weight in Kg.	Total leukocyte number in peripheral blood†
				In lymph	In blood			
FLU	Lymphatic leukemia	2 days	1,100	25,500	135,000	28.1×10^9	59.5	56.2×10^{10}
ELM	Lymphatic leukemia*	6 hours	900	4,800	38,000	4.3×10^9	49.9	13.3×10^{10}
KIR	Lymphatic leukemia	10 days	2,000	5,400	6,000	10.8×10^9	68.0	28.5×10^9
RAL	Myeloblastic leukemia	2 days	1,000	19,500	33,500	19.5×10^9	78.5	18.4×10^{10}
KIN	Hodgkin's disease	3 days	475	200	5,500	9.5×10^7	48.5	18.7×10^9
HAL	Multiple myeloma	12 days	800	2,100	3,500	1.7×10^9	59.0	14.5×10^9

* Right thoracic duct—major lymphatics in the vicinity of the left jugular-subclavian junction had been ligated 48 hours previously.

† Calculated from blood volume estimated at 7 per cent of body weight.

patient (RAL), a 16-year-old boy with a fulminant myeloblastic leukemia associated with chloromatous tumor formation, the cells in the lymph were predominantly small mature lymphocytes which had no consistent numerical relationship to either the total number or differential leukocyte count in the peripheral blood.

The leukocyte content of the thoracic duct lymph in the non-leukemic patients in this study varied from 210 to 5,600 per cmm. In three patients with leukemia the lymph leukocyte count was within the normal limits. In one patient (FLU) it was slightly higher than the upper limits of that reported for normal man (3). The lymph count in these four leukemic patients was approximately 10 to 100 per cent of that in the peripheral blood.

Chemical characteristics. Biochemical studies of simultaneously obtained lymph and venous blood *in vivo* were done on six patients. In four patients, daily or twice-daily samples were studied for 2 to 11 consecutive days (Table IV). The chemical constituents of the lymph and blood remained remarkably constant from day to day during the period of drainage. The globulin content of blood consistently exceeded that of the lymph in all patients and while the albumin content of the blood often exceeded that in the lymph the difference was neither as great nor as constant as that of the globulin (Table V). The total cholesterol content of blood was markedly higher than that in lymph, due primarily to the esterified fraction. The alkaline phosphatase and thymol turbidity determinations of the lymph were found to be 7 and 12 times higher respectively than that of the blood in one patient; in another patient the alkaline phosphatase values for blood and lymph were equal (Table V). The glucose content of lymph in one instance was significantly higher than that of blood.

There was no alteration in the clinical or hematological status, either exacerbation or improvement, in any patient following the procedure.

DISCUSSION

The leukocyte count in the thoracic duct in normal man varies from 2,000 to 20,000 per cmm. (3), consisting almost totally of lymphocytes (Figure 5). This is about 2 to 10 times the number of lymphocytes that can be found per cubic millimeter at any one time in the peripheral blood. The thoracic duct lymph in the normal subject is reported to flow between 1,500 and 2,200 cc. in each 24 hours which calculates to a delivery of approximately 35×10^9 cells per 24 hours into the peripheral circulation (3, 9). Since only one-third of this number can be accounted for in the peripheral blood, it has been assumed that the lymphocyte is replaced three times daily and that the intravascular life span of the lymphocyte can be calculated therefrom (8, 10, 11). Calculations of life span of lymphocytes in the peripheral circulation by this method are based upon the premise of a single securely closed circulation consisting of the flow of lymphocytes from the production site through the thoracic duct into the blood and thence to destruction. There is no consideration of rapid removal by sequestration (12), recirculation in some organ (13), or return of lymphocytes from the peripheral blood back into the lymphatic system (14). If such calculations are valid (and there are many reasons to doubt it) (10), the life span of the lymphocytes in most of these leukemic patients would be expected to be longer than in the

TABLE IV

Biochemical data of blood and lymph during thoracic duct cannulation in patient KIR, m., age 35—lymphatic leukemia

Blood

	5-22	5-23			5-24			5-25		5-26		5-27		5-28		5-29	5-30	5-31	6-2	6-3	6-4	6-5	6-6	6-8	6-12	
	9:00 a.m.	11:30 a.m.	3:00 p.m.	11:00 p.m.	12:30 a.m.	8:45 a.m.	9:00 p.m.	8:00 a.m.	3:30 p.m.	8:40 a.m.	3:15 p.m.	9:00 a.m.	3:45 p.m.	8:30 a.m.	1:30 p.m.	9:00 a.m.	1:30 p.m.	8:30 a.m.	8:30 a.m.	9:00 a.m.	9:00 a.m.	9:00 a.m.	9:00 a.m.	9:00 a.m.	9:00 a.m.	3:00 p.m.
Chlorides	109.9	107.1	107.	112.4		107.	106.2	108.	108.	102.8	105.	104.5	103.6	103.5	103.5	103.5	107.1	104.	103.	106.2	98.2	102.		103.	100.	106.2
NPN	34.8	38.9	33.8	36.8		36.8	35.2	33.8	37.4	32.5	34.0	25.0	23.7	24.3	24.68	27.73	33.4	36.4	31.1	20.4	21.7	30.4	27.8	28.2	23.9	25.7
Total protein	6.31	5.72	6.02	5.57		5.01	4.52	5.16	5.23	5.52	4.94	5.20	4.93	5.2	5.10	5.73	5.40	5.27	5.06	5.82	5.70	5.96	6.17	5.61	5.53	5.13
Albumin	4.07	3.79	3.69	3.38		3.22	2.79	2.95	3.00	2.92	3.02	3.09	2.96	2.7	2.79	3.28	2.92	3.12	2.76	3.10	3.38	3.89	3.42	3.49	3.40	3.13
Globulin	2.24	1.93	2.33	2.19		1.79	1.73	2.21	2.23	2.60	1.92	2.11	1.97	2.5	2.31	2.45	2.48	2.15	2.30	2.72	2.32	2.07	2.75	2.12	2.13	2.00
Sodium	136.7	134.0	129.	133.		130.	130.	130.	133.	138.	142.	36.		132.3		136.5		141.	134.1	131.	137.	135.	135.	131.	131.2	131.2
Potassium	4.7	4.4	4.5	4.9		4.7	4.4	4.9	4.5	4.3	4.9	4.3		4.3		4.9		4.4	5.07	4.7	4.3	4.6	4.5	4.8	3.7	4.5
Uric acid	4.5	4.06	4.10	3.7		3.40	3.46	3.34	3.34	3.20	3.50	3.34	3.50	3.80	3.54	3.34	3.84	4.00	4.16	3.28	3.08	3.92	QNS	4.66	4.04	3.24
Calcium	9.81	8.84	10.42	10.39		8.63	8.74	8.63	9.34	8.63	8.26	QNS	10.70	9.17	8.67	QNS	9.86	8.99	9.33	9.11	9.36	9.91	9.98	9.43	9.47	9.34
Phosphorus	3.25	3.58	4.3	4.4		3.77	3.40	QNS	4.75	3.20	3.82	2.42	QNS	3.92	3.22	3.10	4.77	3.20	3.07	3.15	3.05	QNS	QNS	3.83	3.72	3.96
Glucose			77.0	93.5		82.5		82.8	75.1	71.3	D.C.															
Creatinine	1.11	1.20	1.04			1.13				0.95		0.97		1.10		0.97										
Total	121.		92.5	115.4		100.4	100.4		98.7	103.			106.	92.2		QNS	92.2	97.8	121.4	97.8	123.	121.	QNS	98.51	04.0	75.6
Cholesterol free	28.6	QNS	31.8	27.9		29.7	29.7		25.1	31.9		QNS	37.6	26.7			33.6	28.7	35.6	47.7	46.1	42.2	104.0	42.6	40.6	31.8

Lymph

Chlorides		105.	108.	100.9	104.4	109.	104.4	108.	99.1	105.3		113.3	105.3	105.3	107.1	101.8	98.3	108.	105.							
NPN		39.9	38.7	28.4	39.3	38.1	35.9	36.4	37.44	34.24		26.8	23.7	26.5	24.91	27.86	32.0	33.7	33.0							
Total protein		4.25	3.95	3.76	3.94	2.92	2.72	2.82	3.33	3.00		2.82	2.58	3.21	2.21	3.35	2.85	2.83	3.04							
Albumin			2.62	2.53	2.65	2.02	1.75	1.78	1.98	1.92		1.93	1.79	2.26	1.47	2.14	1.75	1.87	1.97							
Globulin			1.33	1.23	1.29	0.90	0.97	1.04	1.35	1.08		0.89	0.79	0.95	0.74	1.21	1.10	0.96	1.07							
Sodium		142.	137.	133.	135.	136.	135.	136.	133.	136.		141.6		136.3		137.		141.6	123.2							
Potassium		4.7	4.5	4.9	4.8	4.9	4.4	4.7	4.5	4.6		4.8		4.9		5.3		4.1	4.7							
Uric acid			3.90	3.9	3.6	3.46	3.00	2.86	4.30	3.40		3.40	3.16	4.30	3.66	3.30	3.63	3.90	4.24							
Calcium		9.97	8.36	7.92	8.58	7.82	6.96	7.84	8.14	8.26		7.81	7.47	8.28	7.16	8.04	7.95	7.91	8.27							
Phosphorus			3.6	4.1	4.4	3.63	3.41	4.00	4.63	3.42		2.57	3.23	4.25	3.35	3.50	4.79	3.37	3.17							
Glucose			75.6	36.4	95.7	72.0		72.2	77.7	79.0																
Creatinine			1.04			0.97		0.99		1.03		1.02		1.13		1.06										
Total			62.0			63.7	58.4	47.4		50.5			42.2	38.3		52.4	68.0	56.3	66.5							
Cholesterol free			30.2			26.5	21.6	15.9		18.4			23.9	16.4		21.9	41.1	20.7	24.6							

TABLE V

Biochemical data on average blood and lymph obtained simultaneously

		Sodium	Potassium	Chloride	NPN	Uric acid	Creatinine	Total protein	Albumin	Globulin	Cholesterol mg.%		Phosphorus	Calcium	Glucose	Alk. phosphate	Thymol turbidity	Bilirubin	
		mEq./l.	mEq./l.	mEq./l.	mg.%	mg.%	mg.%	gm.%	gm.%	gm.%	Total	Free	mg.%	mg.%	mg.%			Total	Prompt
HAL	Blood	132	5.9	95	141	10.9	9.0	9.4	2.48	6.92	101.4	27.9	6.3	11.8	117				
	Lymph	132	5.6	96	139	10.8	8.9	7.33	2.53	4.80	64.6	26.8	6.4	11.1	136				
RAL	Blood	130	4.6	98	30	3.6	1.4	6.63	3.50	3.13	92.6	30.5	4.4	10.0					
	Lymph	131	4.4	103	26	3.3	1.3	4.25	2.61	1.64	55.6	26.0	3.9	7.8					
BEA	Blood	113	5.0	94	32	4.3	0.8	6.73	3.30	3.43	167	56	3.9	9.8					
	Lymph	118	4.8	87	30	4.1	0.8	4.60	2.40	2.20	98	51	3.6	8.2					
BUT	Blood	135	5.2	98	25.1	5.2	1.0	7.25	3.04	4.21	139	45	4.1	9.8		1.7	5.9	0.6	0.1
	Lymph	132	4.9	99	24.2	5.0	1.0	5.34	2.67	2.67	106	50	3.9	8.2		21.0	42.0	0.9	0.3
KIN	Blood	124	4.1	94	15.8	1.6		5.38	2.0	3.1	83	33	3.7	8.5		7.3		1.1	0.5
	Lymph	124	3.9	95	13.4	1.7		2.91	1.5	1.5	34	15	4.2	6.8		6.3		1.1	0.5

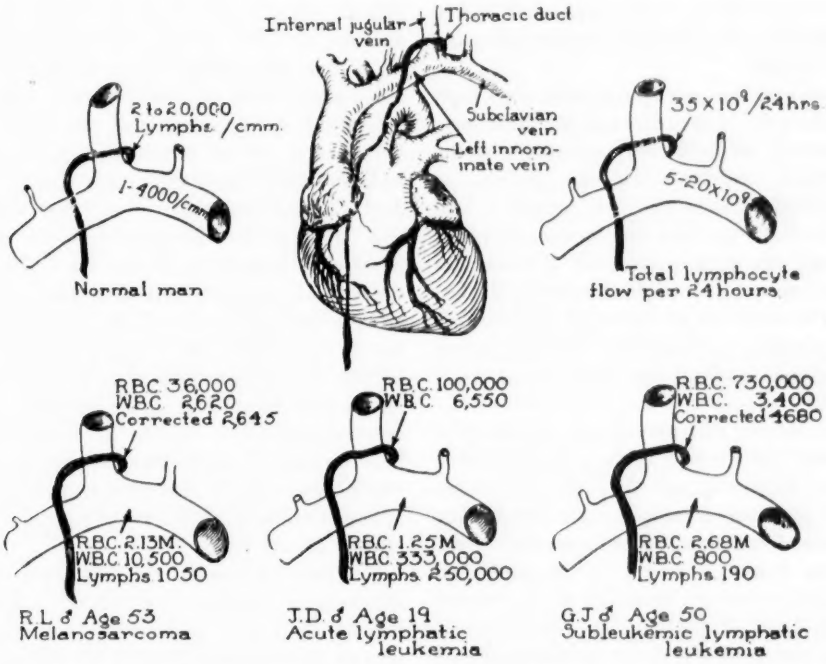

FIG. 5. SCHEMATIC REPRESENTATION OF NORMAL LYMPHOCYTE FLOW AS COMPARED WITH THAT FOUND IN A NON-LEUKEMIC PATIENT, AND TWO PATIENTS WITH LYMPHATIC LEUKEMIA, ONE WITH A HIGH PERIPHERAL BLOOD LEUKOCYTE COUNT AND ONE WITH A LEUKOPENIA

The normal flow of lymph through the thoracic duct in man is estimated at approximately 35 billion lymphocytes per 24 hours. The duct lymphocyte counts in the three patients are approximately the same despite marked differences in the peripheral blood. The corrected counts are those caused by possible dilution with peripheral blood and show little change.

non-leukemic individual (15, 16). This would suggest some defect in the normal destruction or removal of the lymphocyte in the leukemic patient which would be reflected in a longer than normal life span in the intravascular circulation. This prolongation of life of the circulating lymphocyte may reside within the properties of the cell (17), the host or both.

In dogs, rabbits and cats the ligation of both right and left thoracic ducts results in a prompt fall in the peripheral blood lymphocyte number (18, 19). Similar results have been obtained by diverting the lymph flow by thoracic duct cannulation (20, 21, 22). There was no significant fall in circulating lymphocyte number in the two day period of drainage in patient ELM in whom the right thoracic duct was cannulated after the major lymphatics in the left neck had been previously ligated. This would suggest that the life span of the lymphocytes in this patient with lymphatic leukemia was longer than the two days of drainage period or that his major portal of entry of lymphocytes was from a site other than the thoracic duct.

Approximately 5 per cent of all the cells in the lymph in these studies were found to be lymphoblasts which are rarely seen in the circulating blood in the normal individual. This absence of lymphoblasts suggests either a most rapid change within the blood to a small lymphocyte or prompt removal from the peripheral circulation. Similarly, there is a larger percentage of intermediate-sized lymphocytes in the lymph than are found circulating in normal man. It should be noted that the thoracic duct empties just proximal to the pulmonary circulation. Attempts to detect any increases in lymphocyte number by venous catheter sampling at or proximal to the thoracic duct opening met uniformly with failure (12). In the

patients with granulocytic and lymphocytic leukemia, granulocytes were found in considerable numbers in the lymph.

Significant numbers of erythrocytes in the lymph were present only in patients with the leukemias and, in general, reflected the hemorrhagic status of the patient. In those leukemic patients in whom transfusions were given to support a falling blood hemoglobin level, the erythrocyte count in the lymph also rose, particularly if there was little or no improvement of the hemorrhagic component. The factor of an increased permeability of the lymphatics or incompetent vein-duct valves in the leukemic patient also must be strongly entertained.

The data indicate that the number of leukocytes in the thoracic duct lymph in the patients studied usually was materially less than that of venous blood. Of particular significance was this finding in patients with lymphocytic leukemia with either high or low leukocyte counts in the peripheral blood. Similar findings occurred in one patient with myeloblastic leukemia. The non-leukemic patients in this study had fewer leukocytes in the lymph than reported for normal individuals (3, 4). The daily total volume of lymph flowing in the leukemic patients did not exceed that reported in normal subjects (3). Consequently, the total number of leukocytes entering the peripheral blood daily via the thoracic duct in the four leukemic cases in whom accurate lymph flow values were obtained did not exceed 35 billion leukocytes, the value reported by Drinker and Yoffey (3), to be entering the circulation in the normal subject.

The average number of leukocytes in the lymph of eight patients with lymphocytic leukemia, taking the highest count, was 64,600 per cmm. while the peripheral blood at the same time averaged 260,900. The ratio of the counts, lymph to blood, was 1 to 4. Six of these eight patients had lymph leukocyte counts exceeding the 2 to 20,000 per cmm. value reported by Drinker and Yoffey (3). Similarly, in seven non-leukemic patients the highest lymph count averaged 2,760 with the corresponding blood count averaging 6,070—a ratio of 1 to 2.3.

In his original description of leukemia (23), Virchow concluded that leukemia was a disease of overproduction of the leukocytes and shortly thereafter expanded this impression to include the thoracic duct as the route by which the excess number of cells entered the circulation (24). Since this time, many other investigators have accumulated other information which supports the concept of overproduction of leukocytes. These findings are not in accord with this concept of leukemia and create a valid doubt that the thoracic duct is the major pathway of delivery of the excessive numbers of leukocytes into the peripheral blood in all leukemias, or that the leukocytosis in the leukemic patients studied is always a result of an increased production of leukocytes delivered by the thoracic duct route. The possibility of other portals of entry for these cells have not been excluded in these studies. Also, the good possibility remains that there may be a constant variation between rates of production, delivery and removal which may make it fortuitous consistently to fail to obtain evidence of delivery of excessive numbers of cells via any route.

It should be emphasized that these data were obtained on relatively few patients and therefore may not thoroughly represent the variation in lymphocyte flow in man (9). While the numbers of leukocytes obtained in the thoracic duct lymph probably represent the minimum, since in all but one case only the left thoracic duct was cannulated, the avoidance of general anesthesia and the magnitude of the discrepancies between the findings in the lymph and venous blood support these interpretations. The further multiplication of cells by rapid division in the circulation after they leave the thoracic duct requires consideration before any further conclusions can be drawn.

SUMMARY

1. Thoracic duct lymph was collected from 11 cadavers within one hour after death, and from 10 patients during life for continuous periods of 2 to 13 days.

2. The leukocyte count of the lymph varied from 210 to 5,600 leukocytes per cmm. in six non-leukemic patients studied during life.

The lymphocyte count in lymph in the leukemic patients during life usually did not exceed the lymphocyte count in the venous blood. In one patient with subleukemic lymphocytic leukemia for the first of 11 days the leukocyte count of the lymph exceeded that of the peripheral blood.

3. The findings of the studies conducted *post mortem* in 11 patients, 10 of whom had leukemia, were similar to those obtained *in vivo*. In three patients, the lymph leukocyte count exceeded or equalled that in the blood. In the remaining eight patients, six of whom had lymphocytic leukemia, the peripheral blood leukocyte number exceeded that found in the lymph.

4. The erythrocyte number in the thoracic duct lymph obtained from patients with the leukemias was considerable, ranging up to 1.5 million per cmm. The erythrocyte count in the lymph of the non-leukemic patients was generally insignificant. The lymph platelet number varied considerably in both groups (1,000 to 95,000 per cmm.) and never rose to the level found in the venous blood.

5. The chemical composition of thoracic duct lymph closely approximated that of blood plasma with slightly reduced values for albumin and globulin. The antithrombin and prothrombin content of thoracic duct lymph in two patients in whom it was measured was comparable to that found in the venous blood. The cholesterol ester content of the lymph exceeded that of blood.

6. No beneficial or deleterious effects of the procedure were observed on the clinical or hematological course of the patients studied.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Dr. W. O. Reinhardt, Miss Marjorie Brown, Mrs. Oma Mitchell and Mrs. Anna Lance.

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STUDIES IN INTRAVASCULAR COAGULATION. II. A COMPARISON OF THE EFFECT OF DICUMAROL AND HEPARIN ON CLOT FORMATION IN ISOLATED VENOUS SEGMENTS[1]

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(Submitted for publication February 24, 1953; accepted March 25, 1953)

In a previous report (1) a method of preparing isolated blood-filled venous segments in the dog was described. Intravascular coagulation was studied by determining clot appearance, prothrombin consumption, accelerator formation and platelet disappearance within these segments. Of particular interest was the observation that a fibrin clot developed prior to the disappearance of demonstrable amounts of prothrombin or the elaboration of measurable quantities of clot accelerators. Since dicumarol-induced hypoprothrombinemia is currently employed to inhibit or retard intravascular coagulation in man, it became important to ascertain whether induced hypoprothrombinemia actually was associated with retarded fibrin deposition *in vivo* under the same experimental conditions. For purposes of comparison the effect of heparin was also studied.

OBSERVATIONS

Dicumarol was administered orally to 18 dogs. After two-stage prothrombin concentrations (2) were decreased to values ranging from 29 to 2 per cent of the control level, the external jugular and femoral veins were exposed, isolated, and their contents examined as previously described (1). In brief, all demonstrable tributaries to these veins were carefully ligated and cut so that the vessels were entirely freed from surrounding structures. Continuity of each vein with the remainder of the circulation was then interrupted by the careful application of small clamps at each end of the freed segment. At selected intervals vein segments were removed from the animal and by release of one clamp the contents were emptied into clear shallow glass dishes containing 2 to 3 cc. of 2.5 per cent citrate. These dishes were carefully inspected for the presence or absence of a gross clot. Because in untreated animals clotting always occurred in segments isolated for one and one-half hours (1), the failure of a clot to develop within 90 minutes after isolation of a segment was accepted as evidence of clot inhibition.

Since in clinical practice the degree of hypoprothrombinemia is usually estimated by the one-stage method, such determinations (3) also were carried out in eight animals. In each such instance prothrombic activity was reduced to less than 12 per cent. Although in most dogs the degree of induced hypoprothrombinemia was far in excess of that attained therapeutically in man, *in vivo* clotting in the isolated venous segment was not significantly retarded (*i.e.* more than 90 minutes) except in one animal

TABLE I

DICUMAROL DOSAGE	AT TIME VEIN ISOLATED PROTHROMBIN (%)		SPCA	GROSS CLOT WITHIN
mg/kg	2-stage	1-stage	%	90 MIN
17.6	2	1	0	0
13.4	5	5	-	+
18.7	7	-	0	+
4.4	8	13	20	+
17.6	9	2	0	+
18.0	10	4	0	+
3.7	10	-	-	+
9.0	11	-	0	+
1.1	11	8	28	+
18.7	15	-	0	+
7.9	12	-	-	+
18.3	12	-	0	+
9.0	13	9	70	+
13.4	15	-	0	+
9.5	17	12	17	+
3.1	26	-	-	+
7.5	26	-	-	+
4.0	29	-	-	+

[1] This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-220.

(Table I). In this dog the coagulation defect was greater than in any of the other animals: the two-stage prothrombic activity was 2 per cent and the one-stage activity less than 1 per cent of the control values; the clotting time was prolonged to three hours.

The possibility was considered that serum prothrombin conversion accelerator activity (SPCA) had not been adequately depressed by the dicumarol and was responsible for the prompt clotting in the dicumarolized segments. However, estimations of serum SPCA activity by the method of Alexander (4) in twelve of the dicumarolized dogs revealed no SPCA activity in eight (Table I).

The effect of heparin in therapeutic doses was studied in 17 dogs (Table II). In ten of eleven animals, in which the vein was isolated 30 to 60 minutes after the administration of heparin, clotting did not appear within the vein segment for 90 or more minutes. In all eleven animals, Lee-White (5) clotting times at the moment of isolation ranged from four to more than 36 times the control values. When the period of time from the administration of heparin to the isolation of the segment was increased to two hours significant retardation of *in vivo* clotting could still be detected in three of six dogs. In none of these six animals did the Lee-White *in vitro* clotting times exceed twice the control value.

In the dicumarolized dogs there was such extensive oozing into the operative wounds, that it was impossible to maintain adequate hemostasis during the time period necessary for the isolation of the vein segments. Since bleeding to a similar extent did not occur in the heparinized dogs, it was felt that hemorrhage or its sequelae may have been a factor in the observed differences between the effects of the two anticoagulants. Consequently in eight additional animals, in which large doses of di-

TABLE II

HEPARIN DOSAGE MG/KG	INITIAL CLOTTING TIME (MIN)	AT TIME VEIN ISOLATED HRS AFTER HEPARIN	CLOTTING TIME (MIN)	GROSS CLOT WITHIN 90 MIN
2.2	5	1/2	180 +	0
2.2	9	1/2	180 +	0
1.1	13	1	180 +	0
1.1	8	1/2	180 +	0
1.1	10	1	105	0
1.1	11	1	102	0
1.1	17	1	90	+
1.1	10	1/2	60 +	0
1.1	9	1	57	0
1.1	6	1	24	0
0.55	10	1	39	0
1.1	17	2	31	+
1.1	17	2	39	+
1.1	17	2	33	0
1.1	15	2	30	+
1.1	11	2	18	0
1.1	9	2	15	0

TABLE III

DICUMAROL DOSAGE MG/KG	FINAL PROTHROMBIN 2-STAGE (%)	GROSS CLOT WITHIN 90 MIN	HEPARIN DOSAGE MG/KG	INITIAL CLOTTING TIME (MIN)	AT TIME VEIN ISOLATED HRS. AFTER HEPARIN	CLOTTING TIME (MIN)	GROSS CLOT WITHIN 90 MIN
13.4	5	+	0.55	10	1	26	0
					2	23	0
					3	20	+
18.7	7	+	0.55	17	1	60	0
4.4	9	+	1.1	18	1	180+	0
					2	33	0
9.0	11	+	1.1	10	1 1/2	19	0
1.1	11	+	1.1	9	1	78	0
					2	16	0
18.0	12	+	0.55	16	1	25	0
9.0	13	+	1.1	18	1	77	0
					2	24	0
					3	20	+
9.0	17	+	1.1	11	1	162	0
					2	28	0
					3	15	+

cumarol were given and in which the development of clot within 90 minutes was demonstrated, heparin was subsequently administered and additional segments isolated one, two and three hours after injection of the latter drug. In these animals clots did not develop until more than 90 minutes had elapsed in segments isolated one and two hours after heparinization (Table III). Coagulation was not significantly retarded in any vein segments isolated three hours after heparinization, despite the concomitant hypoprothrombinemia. There was, therefore, no evidence that dicumarol had even potentiated the clot retarding effect of heparin.

DISCUSSION

It has been previously reported from this laboratory that only small amounts of prothrombin need be present in the blood to produce a fibrin clot in the isolated vein segment of the dog (1). Data presented in this paper have shown that severe dicumarol-induced hypoprothrombinemia and SPCA deficiency do not retard markedly fibrin deposition in an isolated segment of vein unless sufficient dicumarol has been administered to prolong greatly the *in vitro* Lee-White clotting time of the blood. In contrast, heparin in therapeutic doses significantly retarded intravascular coagulation in similarly prepared venous segments.

It should be clearly understood that these observations do not preclude the possibility that dicumarol retards intravascular thrombosis nor do they demonstrate that heparin is an ideal anticoagulant. Failure to demonstrate clot inhibition with dicumarol may be inherent in the method employed. It can reasonably be argued that slight retardation of clotting in individual segments was overlooked by applying the rigid criteria of 90 minutes delay in gross clotting. Moreover, under the conditions of the experiment the blood in the segment was completely stagnant and isolated from the remainder of the circulation. Finally, the transient anticoagulant effect of heparin was readily apparent. The present study does show, however, that under comparable conditions the anticoagulant effect of heparin is superior to that of dicumarol. Similar findings have been reported by others (6, 7).

There is, of course, ample evidence that dicumarol does exert a retarding influence on blood coagulation *in vitro* (8–10) and on the development of experimental intravascular thrombosis in animals (11–18). In addition, statistical studies in man indicate that the drug produces a significant reduction in thromboembolism (19–21). Bingham, Meyer and Pohle (22) and Rogers, Barrett and Lam (18) found, however, that moderate degrees of dicumarol-induced hypoprothrombinemia did not significantly reduce the incidence of thrombosis in dogs. When the one-stage prothrombic activity was less than three per cent by the method of Quick (23), Rogers, Barrett, and Lam (18) observed not only a marked inhibition of thrombosis but also an important increase in hemorrhagic complications. Richards and Cortell (12) also successfully inhibited intravascular thrombosis with dicumarol dosages that were associated with extensive hemorrhage.

It is important to recognize that there may be a profound dissociation between bleeding and clotting. Our dicumarolized dogs, for example, hemorrhaged profusely during the operative procedures required to expose the vein segments, yet clotting appeared unaffected within the isolated vein. In man, clinical states such as polycythemia vera (24) and thrombotic thrombocytopenic purpura (25) may be attended by both hemorrhage and thrombosis. It has been reported that a prothrombin concentration of between 5 and 10 per cent determined by the method of Rosenfield and Tuft (3) can usually be regarded as safe (26), and that at this level coagulation is retarded (27). What is not known is how effective such levels are against thrombotic disorders.

In the prophylaxis and treatment of thromboembolism there is a constant search for more effective therapy. That thrombosis may occasionally "break through" apparently adequate dicumarol therapy has been reported by Wright (28). Until it is clearly demonstrated that the pathogenesis of thrombosis is the same in congestive failure, in post-operative and post-partum states, in malignancy and in apparently healthy individuals, one cannot assume that hypoprothrombinemia will provide equally effective therapy in all these conditions. It is certainly not easy to translate the results of experimental studies in animals to clinical practice. Nevertheless, the significance of the study herein reported lies in the observation that substances such as dicumarol, which depress that part of the coagulation sequence concerned with

prothrombin conversion, may not provide as optimal an anticoagulant effect as heparin-like compounds which block fibrin deposition through other mechanisms (29–32).

SUMMARY

In experiments designed to study intravascular clotting in the isolated venous segment of the dog, the anticoagulant effect of heparin was found to be superior to that of dicumarol.

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THE EFFECTS OF 1-HYDRAZINOPHTHALAZINE ON CEREBRAL BLOOD FLOW, VASCULAR RESISTANCE, OXYGEN UPTAKE AND JUGULAR OXYGEN TENSION IN HYPERTENSIVE SUBJECTS [1]

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(Submitted for publication August 25, 1952; accepted April 4, 1953)

1-hydrazinophthalazine ("Apresoline") has been shown to lower the blood pressure and augment the renal blood flow in certain hypertensive patients (1, 2). Although the drug induces vasodilatation, its exact mechanism of action requires further study. Some evidence suggests that it may suppress the sympathetic vasoconstrictor reflexes which cause the "overshoot" in arterial pressure following the Valsalva maneuver (3). Also it appears to have the capacity of inhibiting pressor substances of either renal (4) or cerebral (5) origin.

The present study was undertaken in order to measure the effect of this drug, given intramuscularly, on cerebral blood flow, cerebral vascular resistance, cerebral oxygen uptake and jugular venous oxygen tension of patients with essential hypertension. For comparison, other patients were given a placebo. The effects of "Apresoline" have been compared with the changes observed after the placebo and during the hypotension induced by differential spinal sympathetic block (6), 20° head-up tilt (7), dihydroergocornine (8), protoveratrine (9), and sympathectomy (10).

METHODS

1. Choice of subjects: Seven patients with essential hypertension were selected from the medical and surgical wards of the Hospital of the University of Pennsylvania for the "Apresoline" study. Two patients were in the Group I life expectancy classification of Smithwick (11), three were in Group II and two were in Group IV (poorest prognosis).

The placebo injection study group consisted of the following six patients: one in Group I, three in Group II and two in Group IV. Five additional patients were studied after subtotal adrenalectomy (12) (approximately 88–98 per cent) before and after the placebo. The preadrenalectomy grouping of these patients with severe hypertension and vascular complications was: one in Group II and four in Group IV. They were selected at random without regard to their postoperative blood pressure level. All patients were ambulatory and required cortisone or cortisone and desoxycorticosterone at the time of the initial postadrenalectomy study. One patient, I. B. (Table II), was tested 3 and 12 months after operation. These patients were tested to see whether patients with adrenalectomy responded differently after the placebo injection as to mean arterial pressure, cerebral blood flow and oxygen consumption and jugular venous oxygen tension. These patients are shown below the heavy line in Table II.

2. Technique of study: Cerebral blood flow and oxygen consumption were determined in the supine position by the nitrous oxide method (13). Mean femoral arterial pressure was measured through an indwelling femoral arterial needle. Analyses of blood gases were made by methods reported previously (13, 14). Hydrogen ion concentration of arterial and venous blood was measured anaerobically at room temperature and corrected to 37° C. (15). Hemoglobin concentration was measured with the Evelyn colorimeter and the oxygen capacity calculated therefrom. The derived jugular venous oxygen saturation and jugular venous blood pH allowed an estimation of jugular venous oxygen tension from the data of Dill (6, 16). This estimation is assumed to vary directly with cerebral oxygen tension (17). The drug was injected intramuscularly immediately upon completion of the control observations.

3. Dosage of drug and placebo: The dosage of 1-hydrazinophthalazine [2] varied from 0.14 mgm. to 0.36 mgm. per kilogram of bodyweight (total dose 10 to 20 mgms.). Four patients received 14 mgms. or less. The second

[1] This investigation is being supported in part by research grants from the National Heart Institute, U. S. Public Health Service (H-361), and the Ciba Pharmaceutical Products, Inc.

[2] Generous supplies were made available by F. L. Mohr, M.D., of Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

cerebral blood flow measurement was made between 50 and 70 minutes after the intramuscular injection. By this time the arterial pressure had become stabilized at its lowest level or had begun to rise.

The patients in the placebo experiment were given 1.0 cc. of 0.9 per cent NaCl solution intravenously after the control study. The second study of the cerebral circulation was made 60 minutes later. The patients were told that a substance had been given which would lead to a more relaxed feeling. The apparent state of nervous tension of the subject during the initial and subsequent study was estimated.

RESULTS

Tables I and II contain the results obtained in each test. The initial mean values, mean changes and standard deviations of each group are summarized in Table III. The reductions in mean arterial pressure, cerebral vascular resistance and arterial carbon dioxide tension induced by l-hydrazinophthalazine were the only significant changes ($p < 0.02$).

TABLE I

Effects of intramuscular 1-hydrazinophthalazine on cerebral circulation and oxygen metabolism of patients with essential hypertension

Patient	Sex	Age	Smith-wick group	Carbon dioxide								Blood pH 37° C.				Oxygen							
				Content *volumes %*				Tension *mm. Hg*								Content *volumes %*				Tension *mm. Hg*		Saturation *% oxy Hb*	
				Arterial		Venous		Arterial		Venous		Arterial		Venous		Arterial		Venous		Venous		Venous	
				B‡	A§	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
H. R.	F	21	I	56.9	50.8	65.8	55.9	41	34	52	37	7.50	7.57	7.42	7.52	21.1	21.5	12.7	17.5	30	38	61	80
A. C.	F	50	I	53.2	48.6	58.9	50.2	45	36	57	39	7.41	7.48	7.33	7.43	18.7	18.6	12.5	11.8	34	29	61	57
N. M.	F	52	II	47.7	45.6	54.3	50.5	43	37	54	42	7.36	7.43	7.43	7.39	15.7	15.7	10.5	11.6	30	31	60	64
D. F.	M	30	II	46.6	44.9	51.3	49.6	40	39	48	42	7.40	7.40	7.43	7.35	18.2	18.4	12.1	12.5	34	35	61	63
			II	47.5	45.3	54.0	50.5	45	42	48	50	7.33	7.36	7.29	7.31	19.0	19.6	12.7	13.6	38	38	66	66
H. C.	M	44	II	53.2	49.7	58.7	53.3	47	41	60	47	7.36	7.42	7.30	7.37	18.5	18.3	12.4	12.9	41	41	70	74
A. M.	F	39	IV	59.2	55.6	65.3	62.0	49	42	64	56	7.40	7.44	7.31	7.35	15.7	14.8	9.0	8.4				
V. J.	F	47	IV	55.3	52.4	59.6	57.0	41	35	50	41	7.46	7.52	7.39	7.46	17.9	17.7	11.8	12.0	34	31	64	65

TABLE I—*Continued*

Patient	Sex	Age	Smith-wick group	Cerebral										Pulse rate per minute		Respiratory rate per minute		Dosage	
				Blood flow (X)*		Oxygen uptake (X)		Vascular resistance†		Respiratory quotient		Mean art. press. *mm. Hg*						Mgm. total	Mgm. per kilo
				B	A	B	A	B	A	B	A	B	A	B	A	B	A		
H. R.	F	21	I	39	47	3.3	1.9	4.0	3.0	1.06	1.28	155	141	90	105	18	19	10	0.18
A. C.	F	50	I	69	52	4.3	3.6	2.0	2.0	0.92	0.24	137	106	78	84	16	20	12	0.20
N. M.	F	52	II	55	79	2.9	3.9	2.7	1.3	1.27	1.00	146	103	66	76	14	14	14	0.20
D. F.	M	30	II	49	67	3.0	4.0	2.3	1.6	0.77	0.80	113	108	72	69	12	14	10	0.14
			II	77	65	4.9	4.0	1.9	2.0	1.03	0.85	147	131	72	76	16	19	17	0.24
H. C.	M	44	II	40	42	2.7	2.2	3.5	2.6	0.90	0.67	140	108	64	64	16	20	13	0.14
A. M.	F	39	IV	70	61	4.7	3.9	2.1	1.3	0.91	1.00	148	81	87	85	16	16	20	0.36
V. J.	F	47	IV	45	42	2.8	2.4	3.6	3.0	0.70	0.81	162	127	90	114	28	28	18	0.25

* (X) cc. per 100 grams per min.
† Cerebral vascular resistance is mean arterial pressure divided by cerebral blood flow expressed as mm. Hg per cc. blood per 100 grams brain per minute.
‡ B denotes study made before drug injection.
§ A denotes study made after drug injection.

TABLE II

Effects of intravenous placebo on cerebral circulation and oxygen metabolism of patients with essential hypertension

Patient	Sex	Age	Smith-wick group	Carbon dioxide								Blood pH 37° C.				Oxygen							
				Content *volumes %*				Tension *mm. Hg*								Content *volumes %*				Tension *mm. Hg*		Saturation *% oxy Hb*	
				Arterial		Venous		Arterial		Venous		Arterial		Venous		Arterial		Venous		Venous		Venous	
				B‡	A§	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
E. Z.	M	40	I	47.6	47.9	51.5	53.2	45	41	50	54	7.37	7.40	7.34	7.33	17.8	17.7	13.1	12.1	37	34	66	61
B. S.	M	32	II	48.9	51.6	58.0	59.5	45	45	60	56	7.37	7.39	7.29	7.34	20.6	20.1	10.7	11.6	29	30	49	53
W. P.	M	39	II	48.6	48.7	51.6	51.2	46	33	54	40	7.34	7.52	7.31	7.44	20.1	19.7	16.5	17.0	55	53	84	86
M. M.	F	42	II	49.4	50.5	55.0	56.1	42	46	54	55	7.40	7.37	7.32	7.31	17.1	16.5	10.9	10.8	34	35	60	61
G. G.	M	50	IV	51.1	51.3	55.1	55.6	44	39	51	47	7.38	7.43	7.33	7.37	14.1	14.3	9.4	8.9	37	35	64	62
A. F.	F	50	IV	56.2	58.6	62.3	65.0	47	50	54	62	7.40	7.38	7.35	7.32	15.2	14.7	8.7	7.9	32	31	58	54
				51.0	52.9	56.3	57.1	45	49	55	54	7.35	7.36	7.29	7.31	14.8	14.7	9.0	8.4	34	33	58	56
S. W.	M	47	IV	32.5	33.0	38.6	40.2	38	33	45	45	7.26	7.31	7.20	7.24	17.3	17.4	10.4	9.8	37	33	61	58
I. B.	F	44	IV	40.5	41.9	47.2	48.5	31	33	40	41	7.44	7.43	7.38	7.37	14.9	15.2	8.2	8.3	27	29	51	53
				42.2	42.3	48.9	49.7	37	40	44	49	7.37	7.34	7.32	7.28	15.5	14.9	7.9	8.2	30	34	52	58
T. C.	M	43	IV	44.7	44.1	52.8	53.0	46	41	48	52	7.31	7.36	7.26	7.31	19.4	19.5	11.3	11.2	36	34	61	60
E. D.	M	43	II	47.3	47.3	52.2	53.0	42	42	52	50	7.38	7.38	7.31	7.33	18.0	17.6	12.9	12.2	39	36	68	64

TABLE II—*Continued*

Patient	Sex	Age	Smith-wick group	Cerebral								Pulse rate per minute		Respiratory rate per minute		Months after adrenal-ectomy
				Blood flow (X)*		Oxygen uptake (X)		Vascular resistance†		Respiratory quotient		Mean art. press. *mm. Hg*				
				B	A	B	A	B	A	B	A	B	A	B	A	B	A	
E. Z.	M	40	I	95	70	4.5	3.7	1.3^{T}	1.7^{R}	0.83	0.95	127	115	64	68	12	15	
B. S.	M	32	II	49	46	4.9	3.9	2.8^{R}	2.9^{R}	0.92	0.93	135	133	66	60	14	12	
W. P.	M	39	II	32	52	1.2	1.4	5.6^{T}	3.2^{R}	0.83	0.93	178	166	87	83	17	17	
M. M.	F	42	II	80	72	5.0	4.1	1.6^{R}	1.7^{R}	0.90	0.98	125	123	72	72	24	24	
G. G.	M	50	IV	82	67	3.9	3.6	2.0^{R}	2.5^{R}	0.85	0.80	170	170	92	88	24	16	
A. F.	F	50	IV	52	52	3.4	3.5	3.4^{R}	3.0^{R}	0.94	1.05	175	158	80	64	13	16	
				64	54	3.7	3.4	1.8^{R}	2.2^{R}	0.92	0.67	116	121	64	60	16	16	12
S. W.	M	47	IV	49	60	3.4	4.6	2.4^{R}	2.1^{R}	0.88	0.94	116	126	72	78	18	22	15
I. B.	F	44	IV	44	58	2.9	4.0	2.7^{T}	2.1^{R}	1.00	0.96	119	123	80	84	8	8	3
				44	50	3.3	3.4	2.6^{R}	2.3^{R}	0.88	1.09	116	115	76	78	14	20	12
T. C.	M	43	IV	72	38	5.8	3.1	1.5^{R}	2.7^{T}	1.01	1.07	107	102	78	78	16	16	6
E. D.	M	43	II	86	83	4.4	4.5	1.4^{R}	1.4^{R}	0.96	1.06	116	116	64	68	12	15	3

* (X) cc. per 100 grams per minute.

† Cerebral vascular resistance is mean arterial pressure divided by cerebral blood flow expressed as mm. Hg per cc. blood per 100 grams brain per minute.

‡ B denotes study made before placebo injection.

§ A denotes study made after placebo injection.

T indicates that the patient appeared tense during the procedure.

R indicates that the patient appeared relaxed during the procedure.

TABLE III

Mean changes and standard deviation of individual differences in cerebral hemodynamics and oxygen metabolism of patients with essential hypertension after tests with Apresoline and placebo

Significant reductions were observed in mean arterial pressure, cerebral vascular resistance and arterial carbon dioxide tension ($p < 0.02$) after "Apresoline." No essential changes were noted after the placebo injection.

Patient group and number of observations	Mean arterial pressure		Cerebral vascular resistance		Cerebral blood flow		Cerebral oxygen uptake		Jugular oxygen tension		Arterial carbon dioxide tension	
	B	A	B	A	B	A	B	A	B	A	B	A
Essential hypertension with "Apresoline" (8)	144	113	2.8	2.1	56	57	3.6	3.2	36	34	44	38
	−31* ± 20		−0.7* ± 0.5		1 ± 15		−0.4 ± 0.9		−2 ± 3		−6* ± 3	
Essential hypertension with placebo (6)	152	144	2.8	2.5	65	60	3.8	3.4	37	36	45	42
	−8 ± 7		−0.3 ± 1.1		−5 ± 12		−0.4 ± 1.7		−1 ± 2		−3 ± 6	
After subtotal adrenalectomy with placebo (6)	115	117	2.1	2.1	60	57	3.9	3.8	34	33	40	40
	2 ± 5		0 ± 0.7		−3 ± 19		−0.1 ± 1.4		−1 ± 3		0 ± 4	

Units are as in Table I.

* Signifies statistically significant reduction ($p < 0.02$).

Figures in parentheses denote number of observations in each group.

In each group of patients the figures shown represent in the first line under B, the mean values before the injection, and under A, the mean values after the injection. In the second line are shown the mean changes with the direction denoted by plus or minus and the mean changes are followed by the standard deviation of the individual differences.

I. Intramuscular 1-hydrazinophthalazine in patients with essential hypertension

Mean arterial pressure of this group was reduced from 144 to 113 mm. Hg approximately 60 minutes after an average dose of 14 mgms. (Table III). Cerebral blood flow, oxygen uptake and venous oxygen tension were unchanged. No unpleasant reactions were observed. Pulse rate increased. The data of the individual tests are presented in Table I.

II. Intravenous saline placebo

A. In patients with essential hypertension

The data of the individual patients are shown in Table II. Repetition of the observations 60 minutes after the intravenous injection of saline solution showed no significant differences from the initial measurements (Table III). There was no consistent change which could be correlated with the patient's apparent state of nervous tension. Two patients appeared to become more relaxed after the placebo. Vascular resistance increased slightly in one (E. Z., Table II) and decreased markedly in the other (W. P., Table II).

B. In patients who had undergone subtotal adrenalectomy alone or combined with sympathectomy

The individual observations are shown in Table II and the mean changes are presented in Table III. There were no significant changes in the cerebral functions measured in these patients one hour after the intravenous saline injection.

One patient (A. F., Table II) was tested with the placebo injection both before and after adrenalectomy-sympathectomy. Minimal changes following the placebo were observed both before and after operation. Cerebral venous oxygen tension in this patient was markedly stable (31 to 34 mm. Hg).

I. B. was tested three months and again at twelve months after operation. A slight decrease in resistance was observed after the placebo in each study. This patient was tense during the control cerebral blood flow measurement in the early

postadrenalectomy study, but not during either the control or the post-injection measurements made twelve months after adrenalectomy. Resistance decreased more after the placebo in the earlier study. Although the mean change in cerebral blood flow in this group was small and not significant, one patient, T. C. (Table II), had a reduction of flow almost twice the standard deviation of this group. The arterial carbon dioxide tension of this patient was reduced from 46 to 41 mm. Hg as he grew more restless at the end of the 60 minutes. This may account for the reduction in cerebral blood flow (18).

DISCUSSION

Mean values in Table III indicate that cerebral blood flow was unchanged after "Apresoline" at a time when the arterial carbon dioxide tension was significantly reduced. The 25 per cent reduction in cerebral vascular resistance induced by the drug is all the more impressive because the reduction in arterial carbon dioxide tension would tend to increase cerebral vascular resistance (18). The data of Table III show a tendency for cerebral blood flow to decrease after the saline injection in both groups. This was not observed after l-hydrazinophthalazine.

Reubi found the renal vascular resistance to be reduced 40 per cent about 45 minutes after an intravenous injection of 10 to 20 mgms. of l-hydrazinophthalazine (1). The mean arterial pressure of his patients was lower than that of the group we studied, and was reduced by about 15 per cent of the pre-injection level.

Comparative data have been collected on patients with essential hypertension when hypotension in the cerebral arterial system was induced by other drugs (8, 9), sympathectomy (10), or twenty degree headup tilt for 20 minutes. The mean reduction in cerebral vascular resistance induced by l-hydrazinophthalazine appears to be the same (about 25 per cent) as that induced by dihydroergocornine and intravenous protoveratrine.

Reduction in mean cerebral blood flow was observed in patients with essential hypertension only after the extreme pressure drop of differential spinal block. The second flow measurements were made when the arterial pressure appeared to be stabilized, however, and mean oxygen uptake was unchanged. A slight increase in mean oxygen uptake was observed during the head-up tilt. In several of these studies, a reduction in the mean oxygen content of the internal jugular venous blood was observed even though the cerebral oxygen uptake was unchanged (6, 7, 10).

Such observations raised the question as to whether the mean decreases in cerebral venous oxygen content were associated with significant reductions in jugular venous oxygen tension (6). This uncertainty led us to measure indirectly oxygen tension, thus defining the range of "cerebral oxygen tension" and cerebral blood flow in patients with essential hypertension under the conditions of these experiments.

The relatively close agreement of the measurements in the two groups, as indicated by both mean and individual data, is reassuring when one considers the large number of possible biological, experimental and analytical variations. These observations appear to afford a better baseline for the interpretation of real changes in future studies designed to clarify the influence of varying blood gas tensions on the increased cerebral vascular tone of patients with essential hypertension.

SUMMARY

Eight measurements of arterial and internal jugular blood gases, cerebral blood flow, oxygen uptake and venous oxygen tension were made in seven patients with mild essential hypertension before and after a reduction in mean arterial pressure induced by intramuscular 1-hydrazinophthalazine ("Apresoline").

The high cerebral vascular resistance of essential hypertension was reduced. This was associated with a slight reduction in arterial carbon dioxide tension. Cerebral blood flow, oxygen uptake and venous oxygen tension were unchanged after the drug.

The data obtained in the "Apresoline" study have been compared with measurements made before and after a placebo injection in six other patients with essential hypertension. Five patients

who had subtotal adrenalectomy previously were also tested. The observations after the placebo disclosed no significant differences from the initial mean values in each group. Cerebral blood flow and oxygen consumption remained constant.

The reduction in the cerebral vascular resistance of hypertensive patients after "Apresoline" was sufficient to allow cerebral blood flow and oxygen consumption to remain constant and was comparable to that observed during the hypotension induced by dihydroergocornine and protoveratrine.

ACKNOWLEDGMENTS

The authors are indebted to Drs. Charles W. Crumpton, Walter C. Klingensmith and David P. McCallie for assistance in performing some of the experiments. The aid of Drs. Charles C. Wolferth, William A. Jeffers and Carl F. Schmidt in the preparation of the manuscript is greatly appreciated.

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QUANTITATIVE FRACTIONATION OF COMPONENT PROTEINS OF HUMAN SERUM WITH CATIONIC DETERGENTS [1]

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(Submitted for publication February 9, 1953; accepted April 2, 1953)

In a preceding report (1) it was shown that variations in turbidity result when a cationic detergent such as octadecyl dimethyl benzyl ammonium chloride (Octab) [3] is added to serum diluted in a collidine buffer of pH 6.8. These variations were found to be related to protein alterations resulting from acute infections. In acute rheumatic fever, for example (2), serum turbidity with the cationic detergent was greatest during the acute phase of the infection, and during subsidence of rheumatic activity a decreased turbidity resulted.

The present investigation is an extension of a study of the reaction of cationic detergents with different serum protein fractions. It will be shown that a quantitative assay of the alpha globulins, total globulins and the total protein fraction can be made. From these determinations it is then possible to calculate the concentration of albumin and the beta and gamma globulin fractions. This report describes the techniques utilized to produce differential protein precipitation with octadecyl dimethyl benzyl ammonium chloride. Protein assays of sera by Tiselius electrophoresis have been made. Results of the electrophoretic and detergent assays are compared to establish the accuracy of the latter method.

METHODS

The possibility that differentiation of the component proteins of human serum might be accomplished became evident in experiments wherein increasing amounts of diluted NaOH were added to a mixture of diluted serum and a cationic detergent. In such a system there is little or no precipitation when the pH is 4.0. As the pH is slowly increased, there are three distinct increments of density. Between these increments there are intervening plateaus where there is little change in density associated with slight increase in pH. These plateaus develop at pH 7.0 to 7.5, pH 9.5 to 10.0 and pH 11.5 to 12.0. When relatively homogeneous proteins such as fraction IV-1 (containing mostly alpha 1 and alpha 2 globulin), gamma globulin and albumin are employed, it is observed that fraction IV-1 reacts with a cationic detergent at pH 5.8 to 7.0 to form considerable turbidity. Gamma globulin, however, is not reactive in this range of pH provided the protein concentration is below 20 mg. per ml. This protein forms turbidity with a cationic detergent at pH 7.5 to 9.8. Finally, albumin in a concentration less than 20 mg. per ml. is not reactive with a cationic detergent until pH 10.0 is employed. As the pH is increased to 11.0, a sharp increment of turbidity results and at pH 12.0 maximal precipitation results. If sufficient cationic detergent is added to concentrated solutions of albumin or gamma globulin, the effect of pH is not as evident and these proteins precipitate at lower pH levels.

It will also be seen later that NaCl produces an effect on precipitation of the various protein components of human sera. Thus by proper choice of buffers, NaCl concentration, concentration of protein and a cationic detergent, a quantitative assay of the various components might be accomplished.

Preparation of buffers: A. For pH 6.65—A 0.05 M 2,4,6 trimethyl pyridine (collidine) [4] solution of pH 6.65 is prepared (3). To this is added sufficient NaCl to make the concentration of the latter 0.08 M. The pH is again determined on a glass electrode pH meter and is redetermined each day prior to use. If a variation of more than ± 0.05 pH units occurs, the pH is readjusted by addition of 0.1 N HCl, or a new buffer is prepared. *B. For pH 9.6*—A 0.05 M glycine-Na glycine buffer is prepared and adjusted to a pH of 9.6. To this is added sufficient NaCl to make a final concentration of NaCl of 0.08 M. Prior to use, the pH of this buffer is determined on a glass electrode pH meter and readjusted if necessary. *C. For pH 12.0*—A solution containing 0.05 M NaOH and 0.4 NaCl is prepared. The pH of this solution varies between 11.9 to 12.4. On standing, the pH may fall below 11.9 and a fresh solution must then be prepared.[5]

[1] Research carried out under a grant for the study of rheumatic fever from the Masonic Foundation for Medical Research and Human Welfare.

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[3] All of the cationic detergents utilized were obtained through the generosity of Mr. Wellington Rounds, Fairfield Laboratories, Plainfield, New Jersey.

[4] Obtained from Eastman Kodak Co., Rochester, New York.

[5] The exact pH of this solution cannot be determined with a glass electrode because the high sodium concentration makes the electrode insensitive to a pH over 9.0.

Preparation of the detergent solution: One and 0.1 percent solutions of octadecyl dimethyl benzyl ammonium chloride (Octab) are made in distilled water. On standing in the cold the Octab may precipitate from solution. Prior to use, gentle warming of the solution will redissolve the Octab, and the solution will clear.

Preparation of the protein fractions and standard curves: Three human protein fractions,[6] IV–1, II and V, were used to make standard curves for the alpha globulins, total globulin and albumin respectively. These fractions were obtained from the Squibb Laboratories, where they had been prepared by the Cohn fractionation technique. Fractions II and V were electrophoretically homogeneous. Fraction IV–1 contained 74 percent alpha 1 and alpha 2 and 26 percent albumin by electrophoretic analysis.

Fraction IV–1 is readily soluble at 4 percent concentration in 0.9 percent NaCl solution, to form an opalescent solution. After centrifugation only a minute amount of precipitate is removed. The supernatant solution is assayed for total nitrogen concentration by the Kjeldhal method (73 percent by weight of fraction IV–1 was protein material). In calculating the concentration of the alpha globulins, 74 percent of the Kjeldhal protein value is used.

Similar 4 percent solutions of fraction II and V are prepared. Since these solutions are homogeneous by electrophoresis, 100 percent of the Kjeldhal proteins value is used as the concentration of gamma globulin and albumin respectively.

[6] The protein fractions were obtained from Dr. J. N. Ashworth, E. R. Squibb & Sons, Biological and Chemical Laboratories, New Brunswick, New Jersey.

Standard curve for alpha 1 and alpha 2 globulins: The 4 percent solution of fraction IV–1 assayed by the Kjeldhal method is diluted 1 : 100 with collidine buffer of pH 6.65. Aliquots of this fraction, containing between 0.05 to 0.5 mg. of alpha 1 and alpha 2 globulins, are then accurately measured into 15 × 145 mm. colorimeter tubes. Sufficient buffer to bring the volume to 9.0 ml. is then added. To this solution, 1.0 ml. of 0.1 percent Octab solution is added and the tubes mixed immediately by inversion. The turbidity which develops is read on a Lumetron photoelectric colorimeter using a 440 filter. Maximal turbidity usually develops after 12 to 15 minutes. After 20 minutes, the density of the solution slowly decreases.

The optical density, corresponding to the calculated concentration of the alpha 1 and alpha 2 globulins, is then plotted for each determination as shown in Figure 1.

Standard curve for total globulin concentration: A 1 : 100 dilution of a stock solution of fraction II is made in the glycine buffer of pH 9.6 so that each millilitre contains a known concentration within a range of 0.35 to 0.45 mg. per ml. Aliquots of this solution varying in protein concentration from 0.05 to 0.8 mg. per ml. are added to separate colorimeter tubes. To these solutions sufficient glycine buffer is added to bring the volume to 9.6 ml. Four tenths millilitre of 1.0 percent Octab is added and mixed with the solution by inversion of the tubes. Density is then read after 10 to 15 minutes as described above. The optical density for each calculated concentration of gamma globulin is then plotted as shown in Figure 1.

Fraction IV–1 can be similarly made up in glycine buffer and used to make a standard curve for the total globulin concentration. The slope of the curves for fraction IV–1

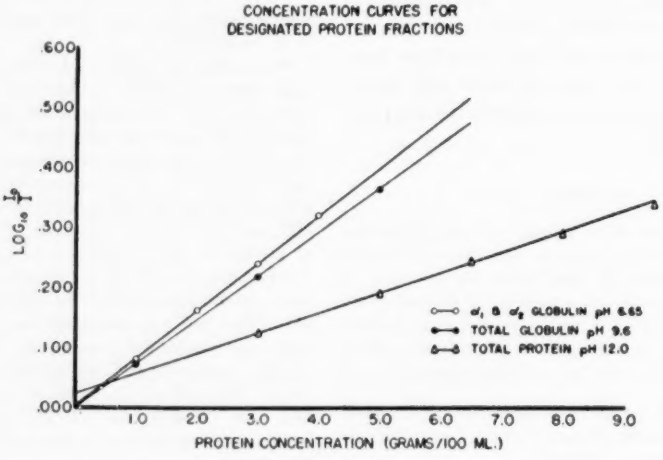

FIG. 1

Standard curves for each protein fraction for the designated pH are shown. The protein concentration is corrected for dilution so that direct reading of an unknown sample can be made. Thus the globulins are 1/100th and the total protein concentration is 1/1000th of the abscissa value shown.

and II is identical. However, as seen in Table I, there is evidence that fraction IV–1, in addition to alpha 1 and alpha 2 globulins, contains an additional small amount of protein reacting at pH 9.6 but not at pH 6.65.

Standard curve for total protein concentration: Albumin is used to prepare a standard curve for assay of the total protein concentration. Fraction IV–1, II or whole serum of known protein concentration is suitable and will produce identical results. A 4 to 5 percent solution of albumin previously assayed by the Kjeldhal method is diluted 1 : 100 in the NaOH–NaCl solution. Aliquots containing 0.04 to 0.08 mg. of protein are pipetted into colorimeter tubes. Sufficient 0.05 M NaOH–0.4 M NaCl is added to bring the volume to 9.6 ml. Four tenths millilitre of 1.0 percent Octab is added and mixed by inversion of the tubes. Density is then read on the colorimeter after 10 to 15 minutes until maximal density is determined. The optical density for each calculated concentration of albumin or other protein solution is then plotted as shown in Figure 1.

The Lumetron model 402–E colorimeter with filter M 440 has been found to produce a steep slope with small increments of density in solutions of protein combined with Octab. Other colorimeters such as the Beckman, Coleman, Evelyn and Klett do not measure such changes as readily, in that slopes such as depicted in Figure 1 have a lessened density gradient with corresponding increases in protein concentration. This difference in instruments is explained by the fact that the Lumetron is designed so that the cuvette is placed about 5 cm. from the photoelectric cell. With this arrangement much of the scattered light does not impinge on the photocell and thus a closer measure of transmission is obtained. In the other instruments mentioned, where the cuvette is placed close to the photocell, much of the scattered light impinges on the photocell, thus decreasing the sensitivity of measurement of turbidity.

Recently, a nephelometric attachment has been used in the Lumetron 402–E colorimeter. Results obtained by this technique indicate that changes in density caused by the reaction of Octab with proteins may be measured with a two to threefold increase in sensitivity over that obtained by the method described above.

A nephelometric technique, or the use of a colorimeter which is designed so that scattered light does not reach the photocell, is necessary, therefore, for an accurate assay of the turbidity developing in the interaction of proteins and cationic detergents.

Assay of mixed protein solutions: An accurately measured amount of serum (0.0500 to 0.1200 ml.) obtained from clotted blood is measured into a test tube. A micropipette has been found to be most suitable. (These may be prepared from 5 mm. glass tubing or they can be obtained from MISCO, Microchemical Specialties Co., Berkeley, California.) Sufficient 0.08 M NaCl solution is added to produce a 1 : 100 dilution of the serum. To assay the alpha 1 and alpha 2 globulin concentration, 1.0 ml. of the diluted serum is pipetted into a test tube. Eight millilitres of collidine buffer solution (pH 6.65) are added. One millilitre of 0.1 percent Octab is then added and mixed with the protein and buffer solution by inversion of the tube. After standing for 10 to 12 minutes the optical density is determined. This reading is repeated until maximal density is recorded. By interpolation from the previously determined standard curve for the alpha 1 and alpha 2 globulins, the concentration of the alpha globulins is calculated.

Total globulin is assayed by adding 1.0 ml. of diluted serum to a test tube. To this is added 8.6 ml. of glycine buffer of pH 9.6. Then 0.4 ml. of 1.0 percent Octab is added to the solution and mixed by inversion of the tube. After maximal density has developed (10 to 15 minutes) the optical density is recorded. By interpolation from the fraction II standard curve the amount of total globulin is calculated.

The assay of total protein concentration is made by addition of 0.1 ml. of the diluted serum to a colorimeter tube. A standardized 0.2 ml. pipette with a curved tip measures accurately this volume of diluted serum. Nine and one half millilitres of 0.05 M NaOH to 0.4 M NaCl solution are added to the tube and then 0.4 ml. of 1.0 percent Octab is pipetted into the mixture. After mixing of these solutions by inversion, density is read on the colorimeter after 10 to 15 minutes. The maximal density is determined and then the total protein concentration is calculated by interpolation from the standard curve.

In most sera, the protein concentration for each fraction assayed is sufficient to cause variation of density ranging from 85 percent to 45 percent transmission of light. In sera where the total globulin concentration or total protein concentration is excessive, then densities exceeding these limits may result. In such instances, the amount of diluted serum used in the test is decreased by a factor of one half. The result obtained from the standard curve is then multiplied by two. In sera with a low concentration of total protein, a more accurate assay results when 0.2 ml. of diluted serum is added to the colorimeter tube. The result obtained from the standard curve is then divided by two.

EXPERIMENTAL RESULTS

It has been stated above that differentiation of the alpha globulins, gamma globulin and albumin can be made, provided the protein concentration, pH and NaCl concentration are suitably regulated. Thus albumin requires a high pH before precipitation occurs, while gamma globulin and the alpha globulins precipitate at intermediate and low pHs respectively. At an intermediate pH (9.6), the alpha globulins combine with Octab in a similar manner to gamma globulin so that a summation of the total globulin fraction results. At a high pH (12.0) all of the component proteins react with Octab and a summation of all proteins results.

In this type of investigation, preparations of "pure" proteins are desirable. Reactions II and V reasonably fulfill this requirement, but it is recognized that even these fractions consist of heterogeneous proteins. Fraction IV–1 is recognized to be sufficiently heterogeneous to introduce error into the quantitative measurements described. An homogeneous preparation of beta globulins has not been available. However, a preparation of fraction III obtained from the Squibb Laboratories is found to react in many respects like fraction IV–1. Since fraction III is not readily soluble in 0.9 percent NaCl and in solution is sufficiently turbid to prohibit electrophoretic assay, no information is available concerning the constituent proteins of the particular fraction. Fraction IV–4 obtained from the same laboratories was studied. The beta globulins contained in it do not react with the cationic detergent at pH 6.65 but behave like gamma globulin.

After comparative studies of sera assayed by electrophoretic and detergent techniques had been carried out (Figures 6 through 8), it was evident that most beta globulins react with a cationic detergent in the same manner as the gamma globulins. This supposition is not always valid as indicated in an investigation of serum from a patient with sarcoidosis of the liver. This lesion had caused intra-hepatic obstruction. By electrophoresis, this patient's serum contained a large beta anomaly in the gamma peak of the descending boundary similar to that described by Zeldis and Alling in patient J. M., page 423 (4). Turbidimetric assay of this serum with Octab revealed an alpha globulin concentration of 2.4 gm. and the beta and gamma fraction was 1.4 gm. By electrophoretic measurement, the concentration of the alpha globulins was 1.40 gm. while the beta and gamma concentration was 4.0 gm. (1.58 beta and 2.46 gamma). Thus a striking discrepancy is found not only within each of the fractions but in the total globulin concentration. The difference in the total globulin concentration (3.8 gm. by detergent assay vs. 5.4 gm. by electrophoretic assay) may be attributable to measurement by electrophoresis of a high lipid concentration. The discrepancy in the assay of the alpha globulins may be explained by the possibility that the abnormal beta globulin found in this serum reacts with the cationic detergent in a manner similar to the alpha globulins.

Effect of NaCl on reaction of Octab with proteins. In a previous report (1) it was shown that the precipitation of serum protein (alpha 1 and alpha 2), with Octab at pH 7.3, is influenced by salt concentration. The effect of NaCl on the precipitation of the alpha globulins, gamma globulin and albumin by Octab has been investigated utilizing pH values of 9.6 and 12.0 (Figures 2 and 3). At pH 9.6 it will be observed that Frac-

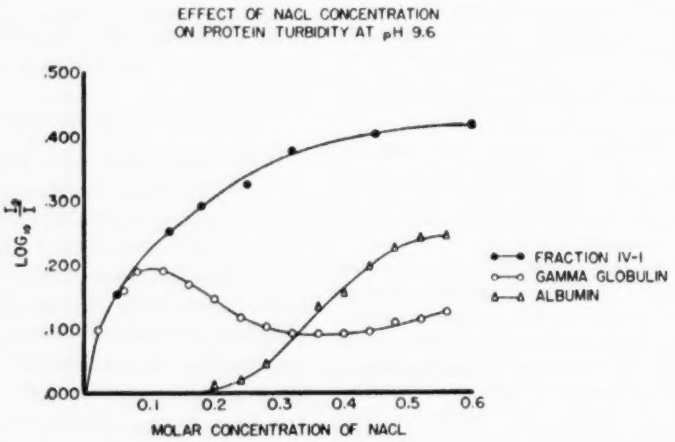

FIG. 2

The effect of sodium chloride concentration at pH 9.6 is shown for each of three protein fractions studied.

tion IV–1 fails to precipitate with Octab if no NaCl is present. An increasing turbidity develops and reaches a maximum in a concentration of 0.4 M NaCl in glycine buffer. Fraction II (gamma globulin) develops maximal density with Octab when the NaCl concentration is 0.08 to 0.12 M. When the NaCl is increased beyond these limits then the density decreases. In the investigations of albumin it is observed that no turbidity develops at pH 9.6 until the NaCl concentration reaches 0.25 M. With the concentration of NaCl increased to 0.5 M, a maximal density results. Since the detergent precipitates in a 0.7 M concentration of NaCl, no higher salt concentrations than 0.5 M were used in this study.

It is seen from Figure 2 that at pH 9.6, alpha 1 and alpha 2 globulins and gamma globulin react similarly if the NaCl concentration is 0.08 to 0.12 M. In this range, albumin is not precipitated. If the NaCl concentration is increased the precipitation of the alpha 1 and alpha 2 globulins is enhanced while the precipitation of gamma globulin is depressed. Finally, with higher concentrations of NaCl, albumin begins to precipitate. For purposes of assay of the total globulins in a mixture of proteins, a NaCl concentration of 0.08 to 0.12 M is optimal.

If these fractions are investigated in a system where the pH is 12.0, a different reaction occurs (Figure 3). It will be seen that NaCl enhances the precipitation of all three fractions to about the same degree. If no NaCl is present, no precipitation occurs. With each increment of NaCl to a concentration of 0.1 M there is a striking increase in density. Beyond this concentration of NaCl, a less steep slope of increasing density develops.

Effect of cationic detergent concentration on protein precipitation. A limitation in the study of the effect of anionic detergents on protein precipitation is imposed by a narrow range of precipitation effect dependent on an optimal protein : detergent ratio (5). This effect is not observed with the cationic detergent Octab when it reacts with albumin, alpha 1 and alpha 2 globulins or gamma globulin at pH 9.6 or 12.0. If the protein concentration is between 0.4 to 0.9 mg. per ml., at least 3 mg. of Octab is required. If greater amounts of detergent are added no change in turbidity results.

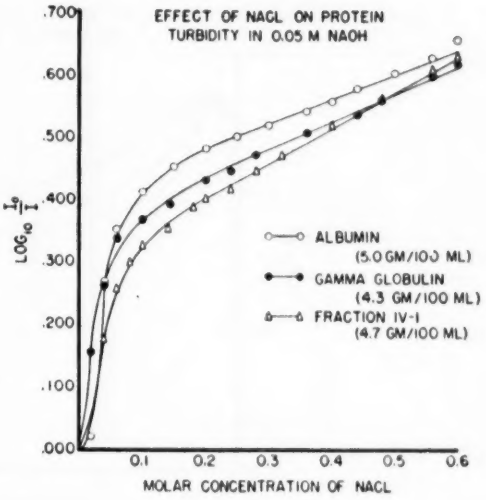

FIG. 3

The effect of sodium chloride concentration at pH 12.0 is shown for each of three protein fractions investigated.

Some influence of the protein to detergent ratio is found however, at pH 6.65, the range employed for alpha 1 and alpha 2 globulin assay. Fraction IV–1, alone and with albumin or gamma globulin added, was prepared in a buffer of pH 6.65 and 0.08 M NaCl concentration. Different amounts of Octab were then added. The data in Figure 4 reveal that the optimal concentration of detergent for the concentration of fraction IV–1 employed is 0.5 mg. per 10 ml. of reacting system (buffer, diluted protein and detergent solution). If the Octab is increased to 1.0 mg. there is a decrease in precipitation. A similar result is obtained in a system which contains fraction IV–1 and albumin. When the combined fraction IV–1 and gamma globulin is added to Octab, however, the decrease in density resulting from addition of 1.0 mg. of Octab is not as great. When Octab is added to a final concentration of 8.0 mg., a considerable decrease in protein precipitation results not only for fraction IV–1 and gamma together but in all the combinations employed.

The sparing effect of gamma globulin on fraction IV–1 precipitation may be related to its ability to combine partially with the detergent at pH 6.65 without being precipitated. Thus the optimal ratio of detergent and the alpha 1 and alpha 2 globulins is maintained. In studies to be described later,

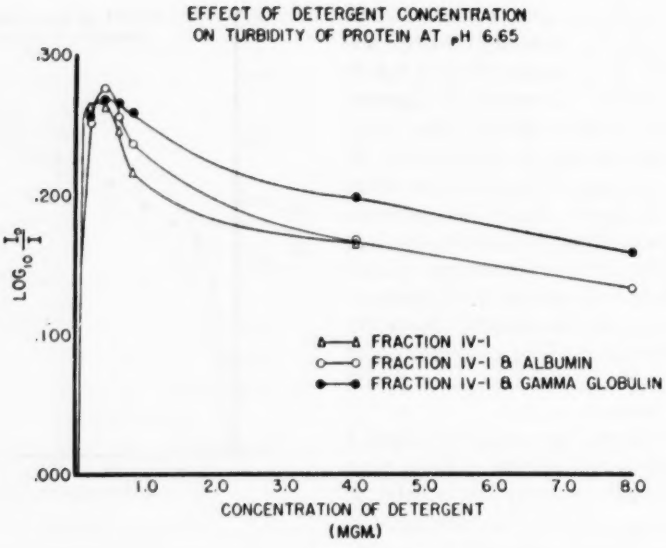

FIG. 4

The influence of cationic detergent (Octab) concentration on combinations of proteins with alpha 1 and alpha 2 globulins (fraction IV–1) is shown.

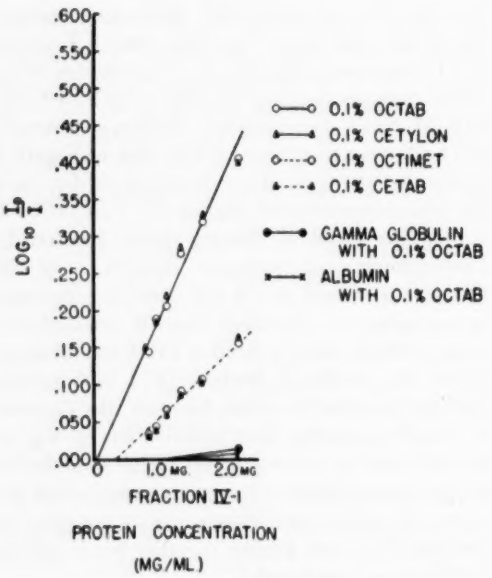

FIG. 5

The effect of different cationic detergents reacting at pH 7.3 in a 0.08 M NaCl solution with fraction IV–1 is shown. Observe that trimethyl or ethyl substituted compounds (Octimet and Cetab) are less reactive than dimethyl benzyl substituted compounds (Octab and Cetylon). Albumin and gamma globulin fail to precipitate under these conditions.

it will be seen that the use of 1.0 mg. of Octab for assay of alpha 1 and alpha 2 globulins permits an accurate determination of this fraction in human serum. In abnormal sera where large increments of alpha 1 and alpha 2 are present with a concomitant depression of albumin and a relative decrease of gamma globulin, the alpha globulins are determined with accuracy. Thus, while it appears that a constant ratio of cationic detergent to alpha 1 and alpha 2 globulins is desirable, such a ratio is not critical when these globulins are present in a complex protein mixture such as exists in serum.

Effect of different cationic detergents on protein precipitation. Four chemically different cationic detergents have been investigated for their reaction with different protein fractions. Octab (octadecyl dimethyl benzyl ammonium chloride) and Cetylon (cetyl dimethyl benzyl ammonium chloride) have identical effects on protein precipitation. They can be used interchangeably for assay of the three protein fractions studied. Octimet (octadecyl dimethyl ethyl ammonium chloride) and Cetab (cetyl trimethyl ammonium chloride) have less effect on protein precipitation. This is shown in Figure 5 where the reaction of fraction IV–1 at pH 7.3 and 0.08 M NaCl is depicted. While a linear relationship between density and

protein concentration exists for each class of detergent, the two with the trimethyl or ethyl substituted groups have a less steep slope than results from the use of the dimethyl benzyl substituted compounds.

If one adds a dimethyl benzyl substituted detergent to a mixture of protein combined with a trimethyl detergent, an increase in precipitation results. Addition of more trimethyl substituted detergent to such a mixture causes no increase in density. Thus, of these four detergents investigated, a long carbon chain substituted with a dimethyl benzyl side chain is more effectively combined with protein than is the trimethyl or ethyl substituted compound.

A chemical analogue of the cationic detergents which lacks the hydrocarbon chain was prepared. This substance p, p'methylene-bis (N_1N-trimethyl aniline) dichloride was added to various protein fractions in solutions with a range of pH from 5.0 to 12.0. No precipitation occurred. Octab was then added to samples to which p, p'methylene-bis (N_1N-trimethyl aniline) dichloride had been previously added and to a control sample. The amount of turbidity developing in each sample was identical. These data suggest that p,p'methylene-bis (N_1N-trimethyl aniline) dichloride neither precipitates nor blocks the precipitation of proteins after addition of a cationic detergent.

If the cationic detergents exert only a physical effect on proteins to produce turbidity it should be possible to remove the detergent by dialysis. Since a 1.0 percent solution of Octab dialyzes through a cellophane membrane, the following experiment was made.

Gamma globulin was treated in the usual manner by precipitation at pH 9.6 with Octab. After standing, the flocculent material was removed and re-suspended in acetate buffer of pH 4.5. The precipitate readily redissolved and the clear solution was subjected to dialysis in a cellophane membrane against a 0.05 M acetate buffer at pH 4.5 for 96 hours at 4° C. Frequent changes of the dialyzing fluid were made during this period. The gamma globulin solution was then brought to pH 9.6 by slow addition of 0.1 N NaOH solution. It again developed turbidity. Such a solution similarly dialyzed and reconstituted in a solution of pH 7.0 and with 0.9 percent NaCl produces hemolysis of erythrocytes which are readily hemolyzed with small amounts of Octab. Thus it appears that little if any cationic detergent was removed by dialysis.

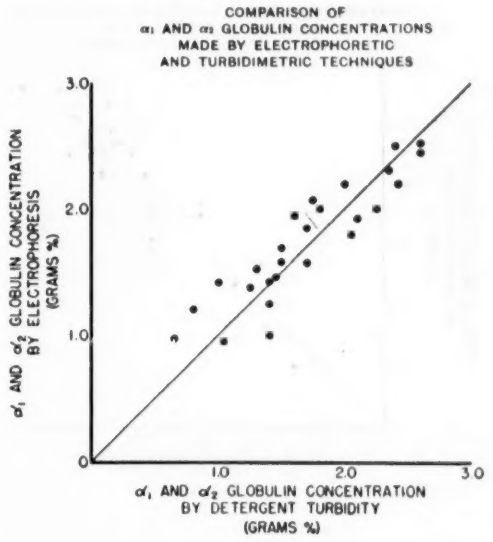

FIG. 6

Comparison of the electrophoretic and detergent turbidimetric techniques for assay of alpha 1 and alpha 2 globulin fractions in serum. The value obtained by electrophoresis (ordinate) is plotted against the value obtained turbidimetrically on the same sample (abscissa). A line of perfect correlation is drawn to orient these comparative results.

Comparative assay of serum proteins by electrophoretic and cationic detergent methods. To determine the reliability of the cationic detergent assay technique in complex protein mixtures such as human sera, parallel studies were made utilizing Tiselius electrophoresis of serum in veronal buffer at pH 8.5. Sera were obtained from patients with such diverse diseases as pneumonia, tuberculosis, cirrhosis of the liver, and so forth. Forty to fifty millilitres of blood were withdrawn and allowed to clot. After centrifugation, the serum was removed and one portion was assayed for the three protein fractions as described above. The remainder of the serum was then suitably prepared and subjected to electrophoresis (6).

A comparison of the two techniques for measurement of the alpha 1 and alpha 2 globulin concentration is shown in Figure 6. Electrophoretic values (ordinate) are plotted for each serum against the corresponding detergent turbidity value

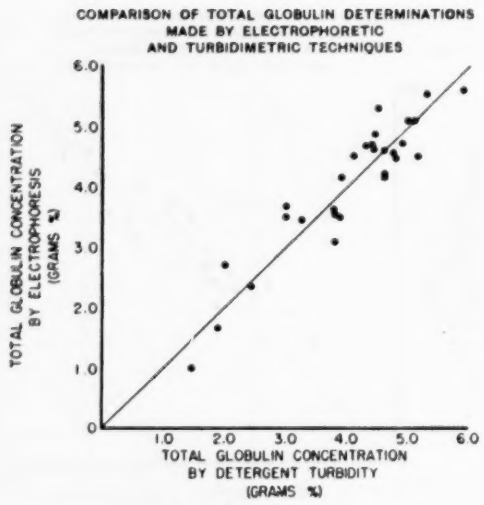

FIG. 7

Comparison of the electrophoretic and detergent turbidimetric techniques for assay of total globulin concentration in serum. Results are plotted in the same manner as in Figure 6.

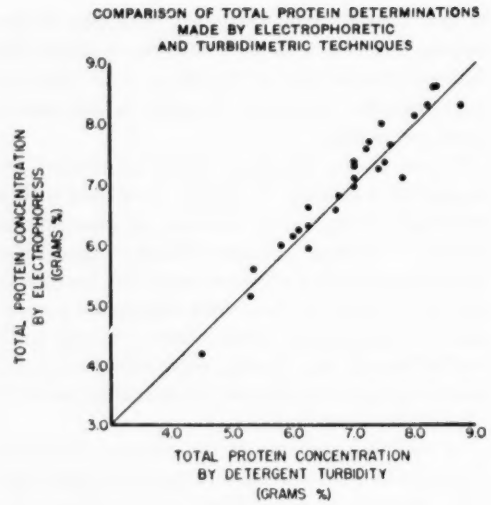

FIG. 8

Comparison of the total protein concentration obtained by electrophoretic and detergent turbidimetric techniques. Results are plotted in the same manner as in Figure 6.

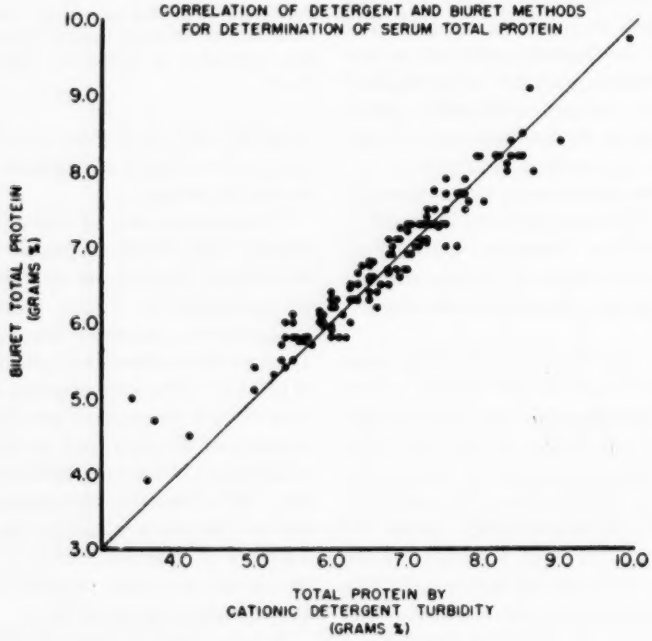

FIG. 9

Comparison of the biuret and detergent turbidimetric techniques for assay of total protein concentration in serum. Results are plotted in the same manner as in Figure 6.

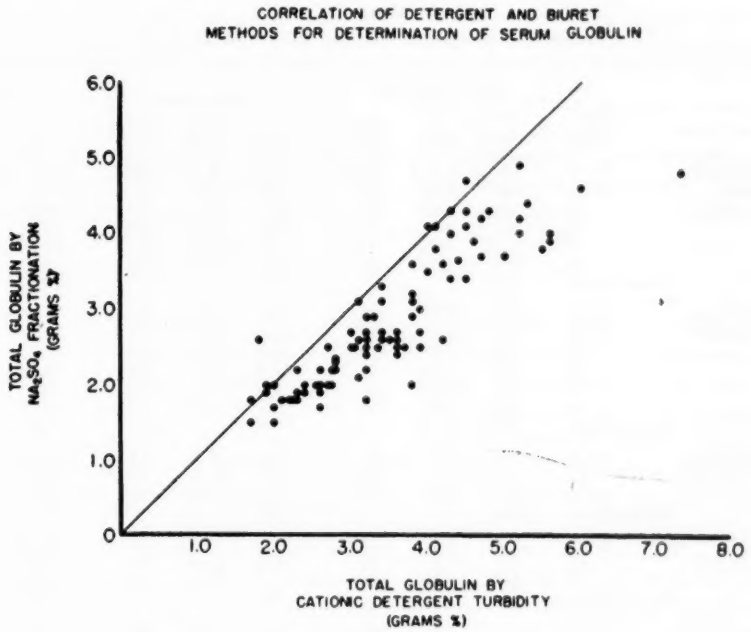

FIG. 10

Comparison of the 22.5 percent Na_2SO_4 precipitation technique with detergent turbidimetric technique for assay of serum total globulin. Observe that, utilizing the latter method, higher concentrations of globulin are consistently found.

(abscissa). A line of absolute correlation is drawn to orient the correlations for each determination. It will be observed that all but five determinations are within $\pm$ 0.25 gm. of the line of absolute correlation ($r = 0.99$).

In Figure 7, similar data obtained for the concentration of total globulin by each method are shown. All but five of 29 sera assayed by the detergent and electrophoretic techniques show a variation less than $\pm$ 0.5 gm. of the value obtained by electrophoresis ($r = 0.70$).

The data obtained for assay of the total protein concentration is similarly shown in Figure 8. All but three determinations were $\pm$ 0.5 gm. of the result obtained by electrophoresis ($r = 0.73$).

A comparison of the assay of total protein by the biuret technique (7) and the cationic detergent technique is shown in Figure 9. In 124 paired determinations on sera from patients, six detergent determinations were more than $\pm$ 0.5 gm. of the value obtained by the biuret method.

Assay of the total globulin was made by 22.4 percent Na_2SO_4 fractionation and subsequent biuret assay of the albumin fraction (7). Aliquots of the same serum were simultaneously analysed for total globulin by the detergent technique. It will be observed in Figure 10 that, utilizing the latter method, consistently higher values of globulin concentration were found. This result is not unexpected since it is known that Na_2SO_4 fails to produce complete separation of the globulins from albumin. Electrophoretic assay of Na_2SO_4 precipitated plasmas reveals that much of the alpha 1 and alpha 2 globulins remain with the albumin fraction (8).

Recovery of protein fractions added to normal and abnormal sera. Sera from a normal individual and from patients with profound alterations of the protein pattern were assayed by the detergent method described. Known concentrations of fractions IV–1, II and V were added to other aliquots of these sera and similarly assayed in order to determine percent of recovery of the added fractions. These data are shown in Table I. It will be observed that serum of patient N. P. has a high globulin concentration but a nearly nor-

TABLE I

Recovery of various protein fractions added to human serum

	Fraction added	Alpha 1 and Alpha 2 globulin			Total globulin			Total protein		
		Found	Predicted	% Recovery	Found	Predicted	% Recovery	Found	Predicted	% Recovery
1. Disseminated Lupus Erythematosis N. P. 1/14/53	IV-1	3.8 g	3.3 g	115%	10.0 g	9.7 g	103%	13.3 g	13.3 g	100%
	IV-1	6.4	5.35	119	14.0	12.0	117	16.0	16.6	96
	II	1.5	1.45	103	10.8	11.2	97	13.5	13.8	98
	II	1.6	1.65	97	15.0	15.0	100	17.0	17.6	97
	V	1.15	1.25	92	7.9	7.4	107	12.25	11.8	104
	V	1.2	1.25	96	8.0	7.4	108	15.0	13.6	110
	Serum alone	1.25			7.4			10.0		
2. Carcinoma of Lung R. S. 3/4/53	IV-1	4.7	4.8	98	9.5	8.1	117	9.9	10.3	96
	IV-1	7.0	6.75	104	11.7	10.4	113	13.0	13.6	96
	II	2.88	2.85	103	10.4	9.6	108	10.25	10.8	95
	II	2.8	3.05	98	13.8	13.4	103	13.75	14.6	94
	V	2.6	2.65	100	6.5	5.8	112	8.9	8.8	101
	V	2.6	2.65	100	6.7	5.8	115	12.25	10.6	115
	Serum alone	2.65			5.8			7.0		
3. Probable Myeloma A. L. 1/27/52	IV-1	4.4	4.5	98	11.6	10.1	115	16.25	16.5	98
	II	1.3	1.3	100	12.8	13.3	96	17.0	17.3	98
	V	0.9	0.9	100	6.4	5.9	108	15.6	15.7	99
	Serum alone	0.9			5.9			10.5		
4. Tuberculosis J. M. 2/3/53	IV-1	5.8	5.6	103	9.6	8.8	109	14.75	15.5	95
	II	2.2	2.4	92	11.6	12.0	97	14.0	14.7	95
	V	2.0	2.0	100	4.1	4.6	89	12.75	13.9	92
	Serum alone	2.0			4.6			8.7		
5. Normal Adult R. A. 2/26/53	IV-1	2.2	2.1	104	4.2	3.9	108	9.6	10.5	92
	II	0.8	0.9	89	5.8	5.7	102	10.6	11.7	91
	V	0.7	0.7	100	2.3	2.3	100	9.8	10.5	94
	Serum alone	0.7			2.3			8.3		
6. Cirrhosis of Liver E. D. 2/15/53	IV-1	3.2	3.05	106	7.9	7.2	110	12.4	11.3	108
	II	1.95	1.85	101	9.2	9.0	102	13.2	12.4	106
	V	1.65	1.65	100	5.7	5.6	102	11.8	11.6	102
	Serum alone	1.65			5.6			9.0		
7. Convalescent Rheumatic Fever J. L. 2/23/53	IV-1	2.8	2.55	109	5.1	4.95	103	9.8	9.6	102
	II	1.45	1.35	107	6.4	6.75	96	11.2	10.7	104
	V	1.1	1.15	99	3.4	3.35	101	10.9	9.9	110
	Serum alone	1.15			3.35			7.3		
8. Rheumatoid Arthritis G. U. 2/19/53	IV-1	3.4	3.35	101	5.4	5.25	103	9.4	10.4	91
	II	2.0	2.05	100	6.9	7.05	98	11.0	11.5	96
	V	1.85	1.85	100	3.7	3.65	101	10.0	10.7	94
	Serum alone	1.85			3.65			8.1		
9. High Fever of Unknown Etiology W. D. 2/14/53	IV-1	5.5	5.4	102	7.0	6.4	109	8.6	8.8	98
	II	3.7	3.6	103	7.6	7.8	98	9.0	9.25	98
	V	3.4	3.4	100	4.8	4.8	100	7.4	8.5	87
	Serum alone	3.4			4.8			5.9		
10. Normal Adult W. O'B. 2/23/53	IV-1	2.2	2.25	98	4.1	4.1	100	10.0	9.6	104
	II	0.9	1.05	86	5.6	5.9	95	10.8	10.8	100
	V	0.8	0.85	95	2.4	2.5	96	9.8	10.0	98
	Serum alone	0.85			2.5			7.4		
11. Multiple Myeloma F. K. 12/12/51	IV-1	10.7	10.65	100	16.4	13.8	119	15.4	16.6	93
	II	9.0	8.85	102	16.6	16.0	104	15.8	17.1	92
	V	8.4	8.6	98	13.8	12.2	112	15.0	15.1	99
	Serum alone	8.6			12.2			13.3		
12. Nephrosis W. B. 1/4/52	IV-1	4.9	5.25	93	6.0	5.5	109	7.3	7.4	99
	II	4.4	3.45	122	7.9	7.7	103	7.4	7.9	94
	V	3.25	3.2	102	3.8	3.9	97	6.5	5.9	110
	Serum alone	3.2			3.9			4.1		

Known amounts of fractions IV-1, II or V were added to different sera. A particular serum and the same serum with the added fraction were then assayed by the cationic detergent technique. The predicted values shown were calculated by addition of the serum value to the value obtained for the particular fraction added.

mal alpha globulin concentration. When two different concentrations of fractions IV–1, II and albumin are added to this serum, 92 to 119 percent recovery of the added protein is accomplished.

The serum of patient R. S., with a metastatic carcinoma of the lung, has a high alpha globulin concentration as well as an increase in the beta and gamma globulin fraction (3.15 gm.). Recovery of added aliquots of fraction IV–1, II and albumin to this serum ranges between 94 to 117 percent.

Patient F. K. is of particular interest, since she had extensive myelomatosis. By electrophoresis, about 60 percent of the total protein migrated in the gamma globulin position. There was little increase in the alpha globulins yet detergent assay reveals a concentration of 8.6 gm. This value represents an extreme elevation and when it is of this magnitude, is diagnostic of multiple myeloma (9). In this instance, there is a marked discrepancy between the detergent assay and the electrophoretic determination. Such a discrepancy is not found in all patients with multiple myeloma studied so far, indicating that only certain myeloma proteins react with a cationic detergent in the pH range of the alpha globulins (9). Despite the abnormal behavior of the gamma migrating myeloma protein, recovery of added fraction IV–1, II and albumin is good.

In patient W. B. with profound serum protein alterations secondary to nephrosis, again, good recovery of added protein fractions is obtained.

DISCUSSION

This investigation of the interaction of the alpha globulins, gamma globulin and albumin with cationic detergents permits three conclusions to be made. First, the magnitude of turbidity produced by the interaction of detergent and protein, as influenced by the hydrogen ion concentration, cannot be related to the isoelectric point of the proteins investigated. Secondly, none of these proteins produce turbidity with a cationic detergent in any range of pH unless NaCl or some similar salt is present in the reaction. In general, an increase in the NaCl concentration causes a proportional increase in turbidity with a cationic detergent when the reaction is carried out at pH 9.6 or 12.0. However, the reaction of gamma globulin at pH 9.6 differs from that of the alpha globulins and albumin in that an increase of salt concentration beyond 0.12 M causes a decrease in turbidity. Finally, the amount of turbidity formed by the interaction of these proteins and the cationic detergents is governed by the molecular configuration of the radicals attached to the hydrocarbon chain. Thus, maximal turbidity develops for a dimethyl benzyl substituted compound, while a trimethyl or ethyl substitution results in a less active interaction of detergent and protein. When a non-hydrocarbon cationic substance, p,p'methylene-bis (N_1N-trimethyl aniline) dichloride is used, neither protein precipitation nor inhibition of precipitation by one of the hydrocarbon cationic detergents results.

It must be recognized that these experiments have not established that turbidity is actually a good measure of protein precipitation. It is possible that the effect of NaCl in particular may not be so much an enhancement of the interaction of protein and detergent but that it may exert some influence upon the size of the agglutinating particles which in turn influence the turbidity of the solution.

There is considerable speculation but little is known about the mode of reaction of anionic detergents with proteins (5). Even less is known about the reaction of cationic detergents with proteins. Under suitable conditions, most, if not all, soluble protein fractions precipitate at or just below their isoelectric points with the anionic detergents (10). In the case of the cationic detergents, it is apparent in a study of albumin in particular, that precipitation occurs at a point considerably beyond the known isoelectric point. Thus it seems likely that cationic detergents may react with proteins by a different mechanism than do the anionic analogues.

The evidence gained by an investigation of the effect of dialysis of the protein and detergent complex and the effects of non-hydrocarbon cationic substance such as p,p'methylene-bis (N_1N-trimethyl aniline) dichloride in neither precipitating nor blocking the cationic detergent effect, does not permit a definitive conclusion regarding the mode of reaction of these detergents with proteins. One can speculate that carboxyl or sulfhydryl groups may combine with the ammonium cationic charges

and effect subsequent precipitation. If one extends such an hypothesis further, one might consider that the alpha globulins have more of these groups reactive at a low pH, while albumin requires a marked decrease in the hydrogen ion concentration before these groups become accessible for interaction with the cationic detergent.

It is apparent that the present data do not allow any reasonable conclusion to be formulated to explain the reaction of cationic detergents with the various human protein fractions under the experimental conditions described. However, a practical advantage of these observations is found when an application of these principles is made in a study of protein mixtures. Thus, utilizing the partitioning effect of a cationic detergent such as Octab, with the pH and salt concentration suitably controlled, it is possible to assay with considerable accuracy the alpha globulins, total globulin and the total protein concentration of human serum. Previous work had indicated that qualitative assay might be accomplished (*2*, 11, 12). The comparative studies of sera by means of electrophoresis and by means of recovery experiments, confirm the specificity of cationic detergent action under an established buffer-salt set-up for each protein fraction.

The use of such an assay technique in clinical medicine has several advantages. Since considerable dilution of serum is required for an optimal detergent interaction with the protein fractions, as little as 0.05 ml. of serum can be employed for an analysis of all fractions. Thus, capillary blood can be used. Secondly, after the preliminary preparation of the buffer solutions and the standard curves, nearly the same information found by Tiselius electrophoresis can be obtained with less effort, utilizing this detergent method. Such a procedure permits a more exhaustive study of protein metabolic alterations in disease and has contributed particularly in investigations of the globulin alterations seen in acute rheumatic fever (*2*), cirrhosis of the liver (9), and other disease states (9, 12).

The plasma protein alterations found in patients with multiple myeloma and in certain types of hepatic disease, produce markedly discrepant results when the electrophoretic and detergent techniques are compared. Further investigation of these divergent findings may reveal additional information concerning the known abnormal character of the myeloma proteins and the beta protein described.

SUMMARY

1. An investigation of the interaction of cationic detergents with three protein fractions, fraction IV–1, fraction II and albumin has been described. The differential effect of pH, salt concentration and molecular configuration of the cationic detergent on protein precipitation is shown.

2. An application of these observations to a differential quantitative fractionation of human serum has been made. By comparative studies with electrophoresis and by recovery experiments, it is shown that assay of the alpha globulins, the beta and gamma fraction, albumin and the total protein can be made with accuracy, utilizing the cationic detergent technique described.

ACKNOWLEDGMENT

Grateful acknowledgment is made to the following: Dr. Eric Alling for the Tiselius electrophoretic determinations reported and for many helpful suggestions made during the course of the investigation; Dr. John Lambooy, who supplied the p,p'methylene-bis (N,N-trimethyl aniline) dichloride; Mrs. Gloria Cushing and Miss Hildegarde Muhlnickel, whose technical assistance was invaluable.

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PEPTIDASES IN HUMAN SERUM: A COMPARISON OF ACTIVITIES BETWEEN NORMAL PERSONS AND PATIENTS WITH LIVER DISEASES AND OTHER PATHOLOGIC CONDITIONS

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(Submitted for publication March 2, 1953; accepted April 2, 1953)

The existence in normal human serum of enzymes belonging to the group of peptidases has been well established in recent years. Grassmann and Heyde (1) first described the hydrolysis of L-leucylglycylglycine (LGG) and L-leucylglycine (LG) by the serums of different species, including man, and attributed these effects to two rather nonspecific enzymes, one a polypeptidase and the other a dipeptidase. Because of the number of different enzymes which have been isolated in recent years from animal tissues (2), it is now generally believed that serum may also contain several peptidases distinguishable by their different specificity patterns.

In most recent investigations the hydrolysis of LGG by serum was compared between normal persons and patients with a variety of diseases. As pointed out by Smith (3), this peptide is particularly ill-suited as a substrate, since it may be attacked by leucine aminopeptidase as well as by

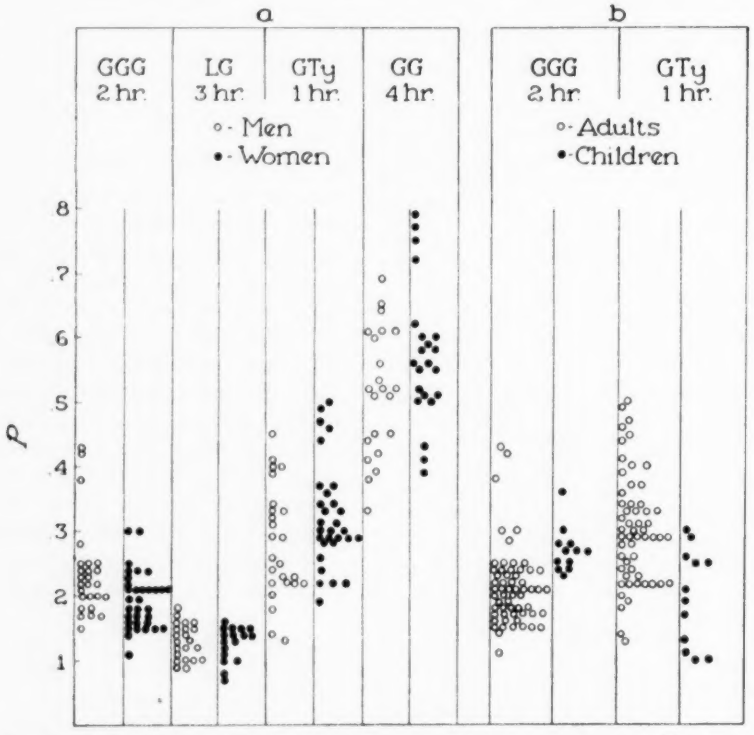

FIG. 1. COMPARISON OF NORMAL SERUM PEPTIDASE ACTIVITIES BETWEEN *a*, MEN AND WOMEN; *b*, ADULTS AND CHILDREN

tripeptidase, both of which appear to be present in human serum. A further complication has often been introduced through the use of cobalt ion which, because of activation of the glycylglycine-splitting enzyme, will cause hydrolysis of the second peptide bond of the tripeptide, in addition to the first. Thus, an increased rate of LGG-hydrolysis in pathologic conditions, as compared with the normal range, may not necessarily indicate an increased tripeptidase activity. The rate of hydrolysis of LGG (or other tripeptides, as indicated) has been reported to be increased in fever (1); fractures (1, 4); lymphatic leukemia, pneumonia, acute rheumatic fever, hypertension, acute barbiturate intoxication (5); lupus erythematosus (with alanylglycylglycine) (6); and in part of the cases of cancer (1, 5); tuberculosis, asthma (5); and acute hemolytic anemia (with glycylglycylglycine) (7).

The present study was undertaken to compare the hydrolysis of four peptides by serum of a group of normal controls with that of a number of patients suffering from a variety of diseases, especially of the liver.

MATERIALS AND METHODS

While no significant change in peptidase activity could be found after a meal (8), fasting blood was used almost exclusively. It was drawn from the cubital vein, and the serum was separated by centrifugation as soon as clotting was complete. The serum was either used immediately or kept frozen at − 18° C. for periods up to two weeks. The freezing had no deleterious effect on the enzyme activities.

Four commercially available peptides were used as substrates. They were glycyl-L-tyrosine (GTy), L-leucylglycine (LG), glycylglycine (GG) and glycylglycylglycine (GGG); their final concentration was 5×10^{-3} M. The buffer system was uniformly 0.06 M veronal-veronal sodium of pH 8.0 (9), which is at or very near the peak of the dipeptidase activities (8). The optimal condition for the tripeptidase of normal serum is approximately at pH 7 (10); however, it was found that in certain diseases, the activity at pH 8.0 is increased more than that at pH 7.0 (8), which has justified the use of one buffer for all four reactions. As shown by other workers (11,

TABLE I

Summary of data of patients with liver disease

Patient	Age and sex	Ascites, grade 0–4	Liver, cm.	Spleen, cm.	Total bilirubin, mg. per cent	BSP retention, per cent	Thymol turbidity	Cephalin flocculation	Alkaline phosphatase	Diagnosis
3	45M	3	5	0	1.6	24	5	—	—	Cirrhosis
9	54M	1	10	0	1.7	30	—	—	—	Cirrhosis
10	64M	0	8	0	1.0	14	—	—	—	Cirrhosis
14	61M	0	6	3	1.7	16	5	3+	3.8	Cirrhosis
17	70M	0	0	0	3.6	40	1	2+	—	Cirrhosis
19	53M	3	3	3	1.7	40	—	—	—	Cirrhosis
21	35F	2	8	6	3.5	22	—	—	—	Cirrhosis
23	41M	1	5	0	5.5	—	15	3+	10.8	Cirrhosis
34	45M	4	0	0	1.8	20	7	4+	2.7	Cirrhosis
39	53M	2	0	0	1.5	32	—	3+	—	Cirrhosis
43	36M	0	6	0	1.3	10	—	—	—	Cirrhosis
44	42M	0	9	0	2.2	32	—	—	—	Cirrhosis
53	59M	0	6	4	1.2	32	—	—	—	Cirrhosis
29	27M	0	4	2	8.2	0	2	Neg.	—	Cirrhosis
15	53F	0	0	0	6.8	—	5	4+	7.3	Post. nec. cirr.
24	73F	2	8	0	1.7	26	8	Neg.	—	Biliary cirr.
26	52M	0	10	4	15.6	—	3	Neg.	32.6	Biliary cirr.
31	43M	0	5	0	12.9	—	4	4+	7.6	Biliary cirr.
33	50M	2	9	5	23.8	—	2	Neg.	18.4	Biliary cirr.
32	40M	1	8	0	0.7	24	4	3+	9.1	Biliary cirr.
6	52F	0	0	0	4.4	20	5	2+	4.6	Inf. hep.
20	76M	0	0	0	20.2	—	9	3+	6.6	Inf. hep.
45	69M	2	2	0	17.8	—	12	4+	4.6	Inf. hep.
46	57M	0	3	0	20.1	—	5	3+	6.6	Inf. hep.
56	25F	0	3	0	14.0	—	4	3+	3.3	Inf. hep.
47	31M	0	10	0	14.4	—	2	Neg.	96.7	Ca. head panc.
25	59M	0	6	0	6.0	—	—	—	—	Ca. head panc.
27	49M	0	5	0	18.4	—	—	—	6.5	Ca. head panc.
28	36F	1	3	0	6.6	—	2	3+	37.7	Ca. head panc.
30	63M	0	3	0	19.4	—	2	Neg.	15.2	Ca. head panc.
55	50M	0	0	0	5.1	—	1	—	13.6	Ca. head panc.
22	38F	0	6	2	23.9	—	4	—	52.9	C.d. stricture
48	47F	0	4	0	2.1	—	2.5	—	26.4	C.d. stricture

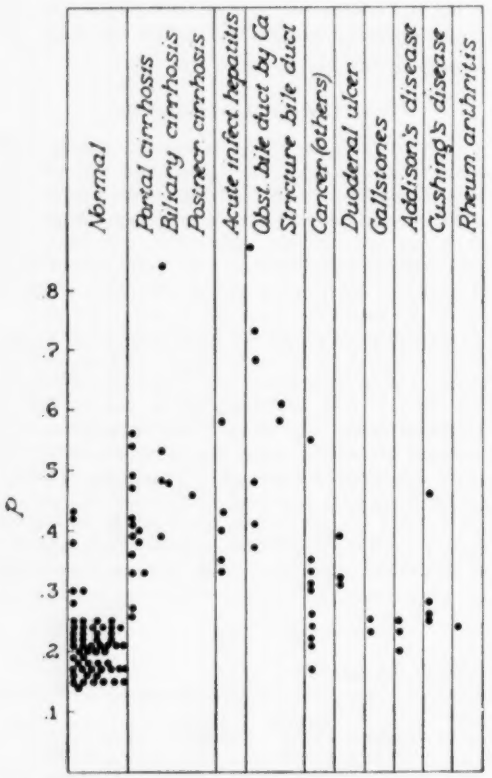

FIG. 2. COMPARISON OF SERUM TRIPEPTIDASE (GGG) BETWEEN NORMAL CONTROLS AND PATIENTS SUFFERING FROM A VARIETY OF DISEASES

12), the tripeptidase is active without any ion added, while the enzyme acting on LG requires manganese (11, 12) (the same applies to the one acting on GTy), and the dipeptidase which hydrolyzes GG has to be activated by cobalt (12, 13). The activation of peptidases by manganese has generally been found to be a slow reaction (2), and this is true also for the enzymes in serum which act on LG and GTy (8). For this reason, serum was first incubated with manganese chloride in the absence of LG and GTy. For each milliliter of serum, 0.5 ml. of 0.1 M veronal buffer and 0.1 ml. of 0.1 M $MnCl_2$ were used. After two hours at 38° C., an additional 2.4 ml. buffer and then 1.0 ml. of 25×10^{-3} M LG or GTy were added to start the reaction. The final concentration of manganese was, therefore, 2×10^{-3} M. Because the activation of the GG-splitting enzyme by cobalt ion appeared to be immediate, a preliminary incubation of serum with cobalt was omitted. The final concentration of $CoCl_2$ was 10^{-3} M.

Hydrolysis was measured by the colorimetric ninhydrin method of Moore and Stein (14), as applied to peptidase reactions by Schwartz and Engel (15). Because of the smaller substrate concentration used by us, 0.5 ml. samples of the reaction mixtures were removed for analysis and precipitated with 2.0 ml. of 1 per cent picric acid solution. The further experimental details were identical with the method of Schwartz and Engel, except that a L-leucine solution of 5×10^{-4} molarity, corresponding to 0.1 μ mole per determination, was used as a reference standard, and corrections were applied to the observed optical densities corresponding to their deviations from Beer's law. Details of this procedure will be described elsewhere (8). The corrected densities were converted to the fractions p of the original substrate concentration which have reacted in the various time intervals. (We prefer to base our calculations on unit substrate concentration rather than on 100 per cent, in order to have a general basis for kinetic considerations.)

A quantitative comparison of p-values can, of course, be made only if a reaction follows zero-order kinetics. This is not true for the tripeptidase, nor does it hold for the other enzymes under the experimental conditions used here; on the other hand, the reactions cannot be adequately described by first-order equations either.

Because of its complex nature, the kinetics of these reactions will be treated in a separate publication (8). In this paper the experimental results will be reported only as the p-values obtained after a fixed time of incubation for each substrate. This was arbitrarily chosen as one hour for GTy, two hours for GGG, three hours for LG and four hours for GG. The findings in the serum of

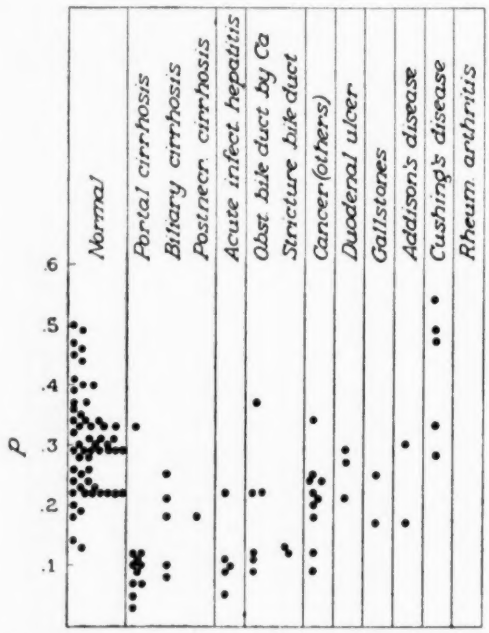

FIG. 3. COMPARISON OF GTy-HYDROLYZING ACTIVITY IN SERUM BETWEEN NORMAL CONTROLS AND PATIENTS SUFFERING FROM A VARIETY OF DISEASES

different individuals will be compared semiquantitatively through the use of scattergrams. Because the kinetics of all four reactions are somewhere between zero order and first order, the statement can be made that differences which appear to be significant in the p-values would be even more so if the proper functions of p were known.

The liver function tests employed were the cephalin-cholesterol flocculation test of Hanger (16), the thymol turbidity test according to Maclagan (17) and the sulfa-bromophthalein test as described by Magath (18). The serum alkaline phosphatase was determined by the method of Bodansky (19), and the serum bilirubin by the method of Powell (20).

The patients studied were all hospitalized and diagnosis in most instances was made by liver biopsy or surgical confirmation except for a few cases of portal cirrhosis in which there were obvious clinical features of the disease with ascites.

RESULTS

In Figure 1*a* a comparison is made between the serum of normal men and that of normal women. There appears to be a slight tendency for the tripeptidase to be higher and for the GTy-splitting and GG-splitting enzymes to be lower in men than in women. If serum of children between the ages of 3 and 14 years is compared with that of adults of both sexes (Figure 1*b*), the tripeptidase seems to be higher and the GTy-hydrolyzing activity lower in children than in adults; however, the data are too few to allow a definite conclusion of this kind.

In Figures 2, 3 and 4 the results of an investigation on three serum peptidases in patients suffering from a variety of diseases are summarized and compared with the analyses on normal serum. The greater emphasis has been placed on liver diseases, since it was noted that in these conditions the activity of the peptidases deviates quite significantly from the normal pattern. For comparison, other pertinent data on the patients with liver disease are summarized in Table I. It will be seen that the tripeptidase activity (Figure 2) is increased in the majority of cases with liver injury. This effect appears to be greater in obstructive jaundice than in diseases affecting primarily the parenchymal cells. However, some elevation of the serum tripeptidase is often found in other diseases.

The data on the GTy-splitting enzyme are listed in Figure 3. It will be noted that this activity is decreased in most cases in which the tripeptidase is elevated, with the exception of the patients suffering from Cushing's syndrome, in which both enzymes are often increased.

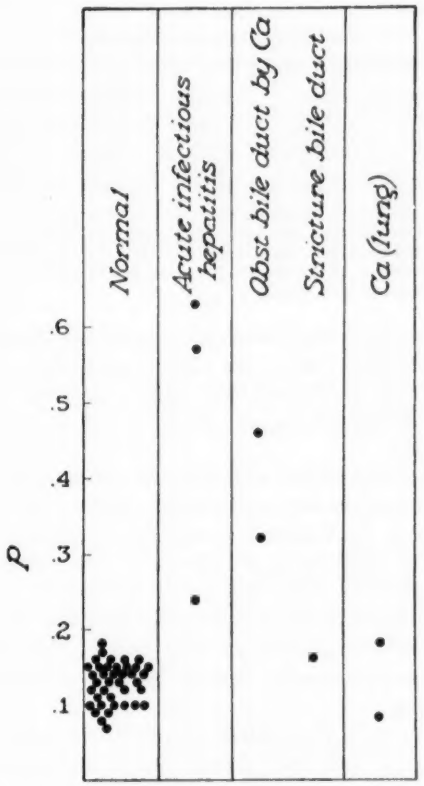

FIG. 4. COMPARISON OF LG-HYDROLYZING ACTIVITY IN SERUM BETWEEN NORMAL CONTROLS AND PATIENTS SUFFERING FROM DISEASE

Data on the LG-hydrolyzing enzyme are very limited but are summarized in Figure 4. Although in normal serum, activity of this enzyme is extremely weak, it was observed to be rather high in five of the six cases of liver disease.

A few observations on the GG-hydrolyzing activity in the serum of patients with liver disease indicate that this enzyme activity may be somewhat decreased, as compared with the normal range.

These results may indicate that the enzymes acting on GGG and LG appear to be increased in liver disease, while those hydrolyzing GTy and GG have a tendency to be decreased.

In three patients with acute infectious hepatitis, serial determinations of the serum peptidases were

TABLE II

*Peptidase activities in human bile**

	Bile a	Bile b	Normal serum	0.9 Serum +0.1 bile b
GGG	.590	.725	.211	.249
GTy	.003	.037	.224	.228
LG	.051	.080	.137	.134
GG	.171	.227	.408	.394

* Two different specimens were used. Specimen b was also mixed with a normal serum to test for synergism or inhibition. All figures are given as p. Conditions were the same as with serum.

performed. The results are recorded in Figure 5 and include data on the alkaline phosphatase and bilirubin [1]; they show that with the recovery from the disease, all enzyme activities tend to become normal.

Another patient suffering from stricture of the common bile duct and secondary biliary cirrhosis showed the expected change of the enzyme activities only during the first week after operation (Figure 6). From then on, the trend was actually reversed in every instance, including that of the alkaline phosphatase. It may be that these data indicate irreparable damage to the liver parenchyma.

The high tripeptidase activity in the serum of patients with liver disease raised the question whether bile itself may contain this enzyme. Table II contains the results of the peptidase test on two specimens of bile which were collected through a T tube several days after a cholecystectomy and choledochostomy. The conditions of the test were in every respect the same as for serum, and the results are also expressed in p. It is interesting that the tripeptidase activity is very much higher than that of the other peptidases and that the GTy-splitting activity is especially low. This suggests that in cases of obstruction, part of the elevated serum tripeptidase has its origin in the bile. The objection may be raised that some constituents in the bile could have an activating effect on some, an inactivating effect on other peptidases. However, a mixture of serum and bile gave results which appear to be about equal to the sum of the activities of the two constituents when tested individually (Table II).

[1] We are indebted to Dr. M. H. Power for the use of these data.

SUMMARY

1. The hydrolytic action of serum on glycylglycylglycine, L-leucylglycine, glycyl-L-tyrosine (both manganese-activated) and glycylglycine (cobalt-activated) was studied in a group of normal persons, in patients with liver disease and in patients with a variety of other pathologic conditions.

2. The greatest changes from the normal range were observed in those patients with disease of the liver. The activity of the tripeptidase was

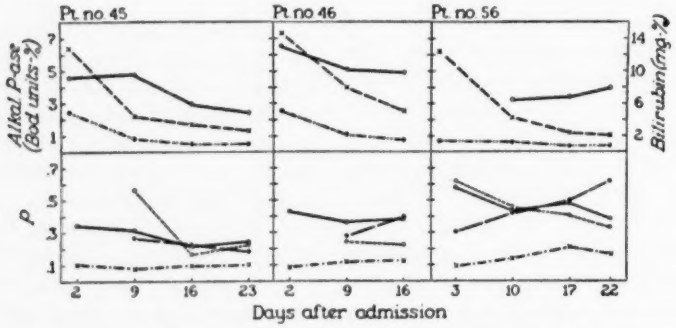

FIG. 5. SERUM PEPTIDASE ACTIVITY IN ACUTE INFECTIOUS HEPATITIS

Lower part, peptidase activities:

○——○ GGG ×– – –× GTy

○– – –○ LG ●— —● GG

Upper part, alkaline phosphatase ○——○ (normal, 5 units per 100 ml.). Bilirubin, direct ×— —× (normal: 0); indirect ●– – –● (normal: less than 0.6 mg. per 100 ml.).

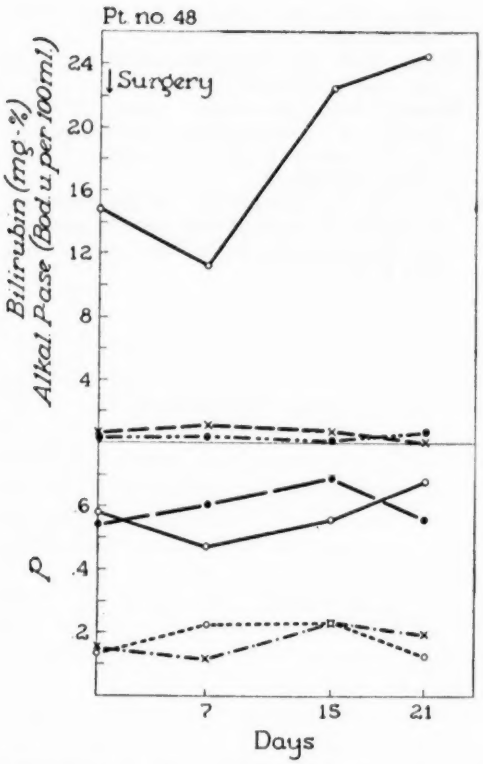

FIG. 6. SERUM PEPTIDASE ACTIVITY IN A CASE OF OBSTRUCTIVE JAUNDICE DUE TO STRICTURE OF THE COMMON DUCT

Lower part, peptidase activities:

O——O GGG O- - -O GTy
×— — —× LG ●— —● GG

Upper part, alkaline phosphatase O——O. Bilirubin, direct ×— —×; indirect ●- - -●.

usually increased. In external hepatic obstruction and biliary cirrhosis, this increase appeared to be greater than in diseases of the liver parenchyma. The hydrolysis of L-leucylglycine, normally of a very low rate, was studied only in a few patients; it too appeared to be high in liver disease. In contrast, the hydrolysis of glycyl-L-tyrosine and glycylglycine showed a tendency to be low in all conditions affecting the liver.

3. The enzyme activities were followed in three patients with acute infectious hepatitis during the initial period of recovery, and a return toward normal was generally observed. A similar study was undertaken on two patients who underwent operation for obstructive conditions of the bile ducts. In one of these neither the peptidases nor the alkaline phosphatase returned to normal within the period of the study.

4. The analysis of human bile revealed a high tripeptidase activity and a low glycyl-L-tyrosine hydrolyzing power. The activities of the other two enzymes were intermediate.

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THE ERYTHEMATOGENOUS ACTION OF ULTRAVIOLET LIGHT ON HUMAN SKIN. I. SOME MEASUREMENTS OF THE SPECTRAL RESPONSE WITH CONTINUOUS AND INTERMITTENT LIGHT

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(Submitted for publication January 8, 1953; accepted April 1, 1953)

INTRODUCTION

A short time after irradiation of the skin with ultraviolet light, an erythema develops. The effective spectral range has been measured by Hausser and Vahle (1, 2) and has been verified by Luckiesh, Holladay, and Taylor (3) and Coblentz and Stair (4). These investigators are agreed that there are two wave length regions of high activity with maxima near 297 mμ and 250 mμ, and one of low activity with a minimum near 280 mμ (see Figure 1). They differ, however, in their reports of the relative activities of the two regions in producing a maximal erythema response. Hausser and Vahle (1) found an almost negligible activity at 250 mμ as compared with that at 297 mμ. Later (2), however, he noted greater activity at the shorter wave length (see curves 1 and 2 in Figure 1). Luckiesh, Holladay, and Taylor (3) found an almost equal activity for both regions (Figure 1, curve 3) whereas the findings of Coblentz and Stair (4) (Figure 1, curve 4), finally, were similar to those of Hausser (2) in 1928.

According to Mitchell (5), the reason for this discrepancy should be sought in the experimental difficulties that arise when working with wave lengths shorter than 280 mμ. The great variability in the sensitivity of the skin to ultraviolet light mentioned in the literature (6) might also hold for the relative sensitivity at different wave lengths and in this way provide an explanation for the discrepancy found by various investigators.

In an effort to localize the erythematogenous processes in the several layers of the skin, we attacked the problem again by irradiating human skin through a quartz spectrograph in order to study the responses for the whole spectrum as did Hausser and Oehmcke (7) who used the skin of bananas. We chose this method because it provided an easy means of comparing the erythematous response of several subjects exposed to different wave lengths.

METHOD

1. *The spectrograph*

For the irradiation of the skin a small f/4.5 quartz spectrograph was constructed consisting of a 60° quartz prism as described by Cornu and two quartz lenses with ± 14 cm. focal length.

The source of light was a high pressure mercury lamp (HP 80 W). To get the highest intensities possible the lamp was placed at the entrance slit. Between the lamp and the collimator lens we placed a series of seven diaphragms. The first of these diaphragms (8×0.5 mm.) was situated at a distance of 5 mm. from the lamp, the others successively farther away and of greater width to ensure a complete filling of the collimator lens. In a like manner five diaphragms were placed between the second lens and the place where the spectrum is formed in order to avoid as much stray radiation as possible.

At the back of the spectrograph, where the spectrum is formed, a quartz object-glass was placed against which the skin could be held.

The casement of the lamp was water cooled and could be moved back and forth for the purpose of adjusting the sharpness of the spectral lines. This sharpness was judged prior to each experiment by means of a fluorescent plate held against the quartz object-glass. The lamp operated both on direct and on alternating current. Although calculated for 80 Watts the lamp could easily be used with 2 Amps.—125 V. to get shorter irradiation times. In order to avoid overheating, the lamp was cooled by a jet of nitrogen from a bomb. Used in this way the lamp could operate at 250 Watts for over 20 hours.

The dimensions of the whole spectrum formed by the spectrograph were 33×8 mm. The surface area covered by the active part (313 − 248 mμ) was 13 mm. long by 8 mm. wide. The smallness of this area has two advantages, first, as several irradiations can be performed on a relatively small skin area there is little risk of local differences in the sensitivity of the skin, second, even heavy doses of ultraviolet light will have no serious systemic effect.

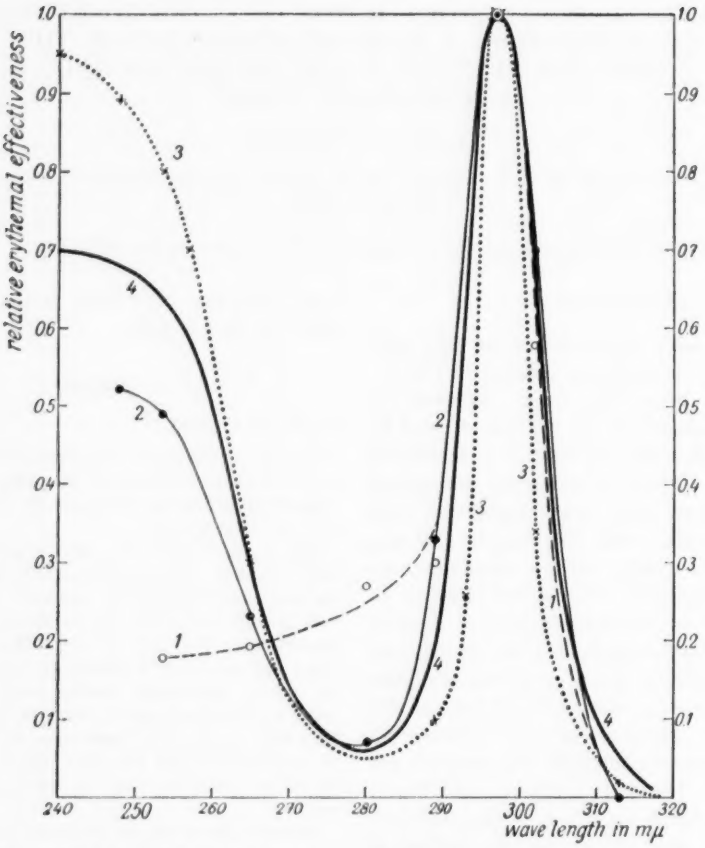

FIG. 1. ERYTHEMA ACTION SPECTRUM AS DETERMINED BY OTHER INVESTIGATORS
Curve 1: Hausser and Vahle, 1921. Curve 2: Hausser, 1928. Curve 3: Luckiesh, Holladay, and Taylor, 1930. Curve 4: Coblentz and Stair, 1934.

2. Determination of the active spectral lines

Irradiation of the skin of the forearm, which is lightly pressed against the quartz object-glass at the back of the spectrograph, for an appropriate time (one to three minutes when the lamp is used with 2 Amps.) leads to the development of an erythema, after six to ten hours, at the sites of the active spectral lines. The determination of the wave lengths by which the erythema is caused is done in the following way. The site of the green line of the mercury spectrum with a wave length of 546 mμ and the short wave length end of the spectrum are marked with two ink lines on the quartz object glass just before placing the skin against it. After the irradiation, the localization of the spectrum is marked on the arm by reprints of the ink lines. The irradiated skin is then outlined with ink. When the erythema has appeared as a red line (8 × 2 mm.) on the skin, the distance of this red line from the site of the 546 mμ line—already indicated in ink on the skin—is measured. The linear distances of all spectral lines from this 546 mμ line have been measured on a photograph of the whole spectrum. Table I gives these distances. With these values the wave length of the erythematogenous line is found.

TABLE I

Linear distances of the spectral lines from the 546 mμ line

Line mμ	Distance mm.	Line mμ	Distance mm.
436	3.0	289	18.8
405	5.0	280	20.5
366	8.2	265	23.8
334	11.9	257	25.7
313	14.9	253	27.3
302	16.6	248	28.3
297	17.5	240	30.8

The spectral lines by themselves are only 0.5 mm. wide but an erythema caused by them is initially 1 mm. wide,

later broadening to approximately 2 mm. At longer wave lengths than 302 mμ an erythema has never been observed. On normal skin the lines 302 and 297 mμ provoke only one red band with a width of 2 mm. This is a conspicuous band and as such is used for the most part as a wave length reference for the other red lines provoked by the shorter wave lengths. Of these the most constantly observed is that provoked by the line 257 mμ (this is not strictly a line but a broad band as is seen in Figure 2). The 257 mμ erythema also occupies a space of approximately 2 mm. Besides this erythema, often those provoked by the 265 mμ and the 248 mμ lines can be observed when they start to develop. Later on these three lines fuse into one broad red patch 5 mm. wide, situated at a distance of 5 mm. from the 302 – 297 mμ erythema. The 253 mμ erythema has not been observed separately.

Curiously enough, in two cases a distinct erythema was observed at the site of the 280 mμ line (between the 302 – 297 mμ red patch and that of 265 – 248 mμ), where the erythematous activity should be very low. In these two cases exceptionally high doses had not been used, so that an abnormal sensitivity of the skin must have existed. This phenomenon has not been studied further.

3. *Evaluation of the spectral response*

We have restricted our attention to a more or less quantitative evaluation of the two principal erythematous

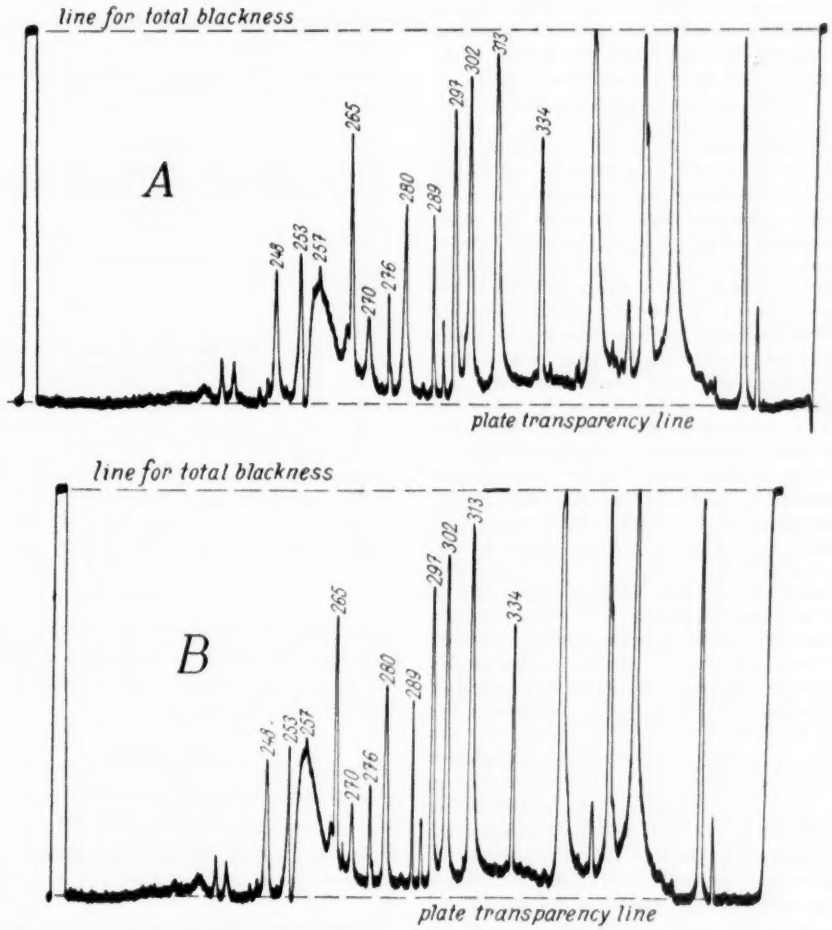

FIG. 2. MICROPHOTOMETER REGISTRATIONS OF THE SPECTRUM OF THE MERCURY LAMP (2 AMPS)

A: alternating, B: direct current. Only the region near 257 mμ is significantly different in intensity. (As both curves are photographic magnifications of the original registrations to obtain equal height for equal intensities, the wave length scale has become different.)

responses at the wave lengths of maximal activity, *viz.* at 302 – 297 mμ and at 265 – 248 mμ.

There are two different ways to judge the effect of ultraviolet irradiation of the skin: (*1*) by measuring the degree of redness for a given irradiation dose or, (*2*) by measuring the irradiation dose which provokes a just-perceptible erythema.

The first of these methods is the less applicable to our purpose. An erythema develops slowly and reaches a maximum 10 to 14 hours after the irradiation. It is not easy to find an irradiation time which produces a maximum redness just matching a given color standard. There is no linear correlation between irradiation dose and degree of redness. Therefore interpolation between two color standards of different redness is not accurate. Moreover erythematous responses to different wave lengths do not change in proportion to identical increases in irradiation doses. The longer the wave length, the greater is the increase in redness with heavier doses (Hausser [2]).

For these reasons we chose the second method of comparing the two responses under discussion, *viz.* by measuring the irradiation dose which provokes a just-perceptible erythema. This method is easily performed as will be clear from the description in the following section.

Both methods have been employed for the determination of the relative effects of irradiation with light of different wave lengths. The first method was used by Hausser (Figure 1, curve 2), the second by Coblentz and Stair (Figure 1, curve 4), without any great difference in result. This agreement may have been due to chance.

Erythema is the result of the widening of the subepidermal capillaries, which vary greatly in number per unit area of skin. The entire area covered by these capillaries is a relatively small fraction of the total area. The color of the skin is determined by a mixture of light reflected from the external surface of the skin and light thrown back after refraction in the epidermal cells and the subepidermal tissue (8). Part of the refracted light passes through the capillaries and the deeper vessels, thus giving a reddish component to the color of the skin. With erythema only a small fraction of the light is changed by the dilated capillaries. The rest of the light that determines the color of the skin remains unaffected, unless fluid passes through the capillary walls, possibly changing the light diffusing properties of the surrounding tissues. Especially with more intense erythemata this may confuse the issue.

Accordingly, the only correlation between irradiation dose and degree of redness of the skin is an increase in the redness with increasing doses, but this increase in redness is difficult to calculate. Hausser estimated the degree of redness of the 297 and 250 mμ erythemata by comparing them with pieces of paper colored with different quantities of a red dye. He found that the 297 mμ erythema matched a paper containing twice as much dye as the paper that matched the 250 mμ erythema (which had been produced by the same energy as the 297 mμ erythema). His conclusion that irradiation at 297 mμ induced capillary dilation two times greater than that at 250 mμ, or that a double stimulus strength had been in operation, seems inadmissable. On the other hand, the spectral response determined by measuring the minimum erythema dose (MED) will be quite different for different skins for the reasons stated above. Thus, *a priori*, it is not to be expected that both methods will give the same results when used on different skins.

Therefore, we are of the opinion that the different action spectra, found by different investigators, as shown in Figure 1, are not due to experimental difficulties encountered with ultraviolet light of short wave lengths (Mitchell [5]), but might be physiologically significant.

4. *Determination of the minimal erythema dose*

As has been discussed in the preceding section we determined the irradiation times necessary to provoke a just perceptible erythema with the whole spectrum of the spectrograph. The procedure adopted was as follows. On the skin of the forearm three or four doses were given by increasing the duration of the subsequent irradiations. From about the fifth hour the irradiated spots were observed at hourly intervals and developing erythemata noted with the following signs:

TABLE II

Minimal erythema dose (MED) for the two wave length groups: 302–297 mμ and 265–248 mμ (2 Amps.,)

Time elapsed since the irradiation	Irradiation time in seconds							
	45		60		75		90	
	300	250	300	250	300	250	300	250
hours								
5	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	±	–
8	–	–	–	–	±	–	+	±
9	±	–	±	±	+	±	++	±
10¼	±	±	+	+	++	+	++	++
11	±	±	+	++	++	++	++	++
12¼	±	+	++	++	++	++	++	++
13¼	±	+	++	++	++	++	++	++
14¼	±	+	++	++	++	++	++	++
23	–	–	–	–	++	±	++	+

– no erythema
± reaction uncertain
\+ just perceptible
++ distinct erythema

From the complete list of observations the minimum erythema dose is easily computed as is shown in Table II.

In this way careful observation every hour makes it possible to determine the MED with an accuracy of approximately 20 per cent. After some experience two or three irradiation doses should suffice to obtain this accuracy.

TABLE III

Minimal erythema doses (MED) with irradiation using direct or alternating current*

Person			Skin area used	Wave length group	MED (alternating current)	MED (direct current)
No. and sex	Age	Skin tan				
				mμ	sec.	sec.
1 ♂	±60	none	fore arm	300	70	95
				250	70	70
2 ♂	±60	moderate	fore arm	300	90	90
				250	300	150
3 ♂	±25	none	fore arm	300	50	65
				250	50	50
4 ♀	5	strong	shoulder	300	180	300
				250	240	420
5 ♀	12	none	shoulder	300	30	30
				250	60	60
6 ♀	11	none	shoulder	300	90	120
				250	150	120
7 ♀	7	none	shoulder	300	50	50
				250	90	50

* The source of irradiation was a lamp operating on both direct and alternating current of equal strength (2 Amps., 250 Watts).

5. *Mechanical influence on the sensitivity of skin*

Experiments that might show the presence of a photosensitive substance on the skin were performed by washing the skin before irradiation with cotton soaked in alcohol or ether, and with brush and soap. These manipulations caused a distinct increase in the degree of erythema as compared with the degree of erythema in the untreated skin. Even rubbing the skin with a finger for some minutes until a distinct redness had appeared showed this effect. These experiments demonstrate how varying degrees of vasolability may influence the apparent sensitivity of the skin. During experiments for determining sensitivity, care must be taken, therefore, not to irritate the skin mechanically.

RESULTS AND COMMENT

Table III shows the result of MED determinations for seven persons, with the lamp burning on direct as well as on alternating current, both of equal strength (2 Amps.).[1]

[1] The voltage depended somewhat on the lamp used; values from 124 to 132 V have been found. Thus different lamps could consume from 228 to 264 Watts. As each experiment was completed with the same lamp, these differences of less than 10 per cent from the mean of 246 Watts have not been taken into account.

As will be seen from the figures of Table III we found, as did all other authors, very different values for the MED for different persons. This statement also holds for the 302 – 297 mμ group as well as for the other and there is only a very slight correlation between both groups.

Stress should be laid upon the fact that these MED values are by no means definitive or characteristic of these persons, as other values may be found at other times.

Reversal of the MED of the two wave length groups with direct current

When we used the lamp with alternating current—as we did in our first experiments—the MED of the 300 mμ group was always found to be shorter in seconds irradiation time than that of the 250 mμ group. The 300 mμ erythema usually appeared first and was the strongest for a given irradiation time, as is illustrated by subjects 2, 4, 5, 6, and 7 (Table III). But later, when direct current was used, a reversal in the time of appearance and in the intensity of both erythemata was sometimes noted. Subjects 1 and 3 (Table III) showed this reversal of the MED with direct current. With these two individuals the use of direct current required a longer irradiation time to provoke the 300 mμ MED. This reversal in the appearance of the two erythemata after irradiation with direct current had nothing to do with a difference in sensitivity to ultraviolet light. From subjects 2, 4, 5, 6, and 7 (Table III), who did not show the reversal, subject 5 was more sensitive than subjects 1 and 3, who did show the reversal, subject 7 had almost the same sensitivity, subjects 2, 4, and 6 were less sensitive.

Comparison of the activity of each wave length group for direct and alternating current

When a certain MED had been found for the 300 mμ group with alternating current it follows from the figures of Table III that the MED in seconds irradiation time for direct current could be longer (subjects 1, 3, 4, and 6) or equal (subjects 2 and 5) but not shorter. Thus, one may conclude that the 300 mμ group is more active with intermittent light than with continuous light.

No such rule has been found for the 250 mμ group. The MED for subjects 2, 6, and 7 was

shorter for direct current than for alternating current, equal in subjects 1, 3, and 5, and longer in subject 4. Thus no final conclusion can be drawn as to the influence of intermittency of irradiated light.

From these observations it seems to follow that there is an unmistakable influence exerted by the kind of current used, which, in turn, determines the type of light produced, intermittent or continuous. Before drawing this conclusion, however, it is necessary to measure the intensities radiated by the lamp with both types of current. It is well known that the relative intensities of the spectral lines depend on the conditions under which a mercury lamp works. Thus it could be possible that our lamp emitted different intensities in the two active wave length groups when used with direct or with alternating current. In the following paragraphs we have described the experiments that were done to clarify this point.

The relative intensities of the spectral lines

In order to measure the intensities of the wave lengths emitted by the HP 80 W, the spectrum was calibrated photographically in the following way.

The lamp was placed 3 meters in front of the spectrograph. At the original site of the lamp an entrance slit 2×0.1 mm. was fitted. Close to the entrance slit a shutter device was placed which permitted a range of exposure time from 0.01 to 9.00 seconds with an accuracy of better than 0.001 second. This shutter device consisted of an aluminum disk with an adjustable sector opening and an automatic shutter rotated by a small synchronous motor. The photographic plate (6×9 cm.) could be moved up and down in the usual manner, so that there was ample space on each plate for 14 photographs of spectra—each 2 mm. high. Seven spectra—each time using a double exposure time—were taken of the HP 80 W alternating with seven spectra of an absolutely calibrated Krefft standard lamp (9), situated at the same place as the HP 80 W. The densities of the spectra were registered photographically with a microphotometer (type C of Moll, N. V. Kipp & Zonen, Delft). An example is given in Figure 2. From these densities the intensities of the spectral lines were derived graphically in the usual manner. The intensity of the continuum was also measured and taken into account.

This calibration was done for the HP 80 W burning on direct current and on alternating current (50 cycles per second). First, a possible intermittency effect on the photographic plate was studied by taking seven spectra of the Krefft lamp burning with 2.0 Amps. direct current (for which this lamp had been calibrated) and then by taking seven spectra with double exposure times through a rotating sector disk giving 50 interruptions per second, each of 0.01 second. On Gevaert Replica 23° S an intermittency effect could not be found with certainty and thus has not been taken into account.

Table IV shows the total intensities for several spectral lines of the HP 80 W with both direct and alternating current. As is seen in Table IV the intensities of the lamp burning with alternating current are a little lower (mean, 4 per cent) than with direct current. Although the accuracy of the photographic method is of the order of ± 5 to 10 per cent and thus does not permit a final conclusion, this difference of 4 per cent might be significant because the lower temperature the lamp will attain with alternating current falls to zero 100 times a second.

For our purpose the conclusion may be drawn that the intensities of the lamp for both types of current are the same. This holds also for each spectral line, especially those that can provoke an erythema (**boldface** in Table IV) if they have sufficient strength. Only the line with wave length 257 mμ is an exception since it provokes a significantly more intense response with direct current than with alternating current. This is a relatively weak line superposed upon a strong continuum of the 253.7 mμ line which itself shows

TABLE IV

*Total intensities (line plus continuum) of the high pressure mercury lamp—HP 80 W (Philips)**

Wave length	Total intensity		Difference
	Direct	Alternating	
mμ	*mW/sterad*	*mW/sterad*	%
578	896	905	− 1
546	1025	954	+ 7
436	755	705	+ 7
408	76	72	+ 5
405	386	366	+ 5
366	945	957	− 1
334	85	80	+ 6
313	552	528	+ 4
302	240	237	+ 1
297	159	154	+ 3
289	68	60	0
280	90	84	+ 7
265	176	180	− 2
257	85	72	+15
253	72	68	+ 6
248	69	64	+ 7

* This lamp burned with 2.0 Amps. direct and alternating current. The lines with strong erythematous activity are printed in boldface.

TABLE V

Minimal erythema dose calculated on basis of radiation intensity and time

Person	Spectral line	Minimal erythema dose mW/sterad × seconds		Alternating current/direct current	Sensitivity	
		Alternating current	Direct current		Alternating current	Direct current
	mμ				%	%
1	297	1080	1500	0.72	1.14	1.13
	257	504	594	0.85	1.96	1.91
2	297	1380	1430	0.97	0.89	1.19
	257	2160	1280	1.69	0.46	0.88
3	297	770	1020	0.76	1.60	1.66
	257	360	425	0.85	2.74	2.66
4	297	2770	4760	0.85	0.44	0.36
	257	1730	3660	0.47	0.57	0.31
5	297	460	476	0.97	2.67	3.56
	257	432	510	0.85	2.29	2.22
6	297	1380	1900	0.73	0.89	0.89
	257	1080	1020	1.06	0.92	1.11
7	297	770	793	0.97	1.60	2.14
	257	647	425	1.52	1.53	2.66

complete self-reversal in the HP 80 W. As can be seen directly from the photographic registrations of the spectra of this lamp (Figure 2) the only real difference is just this continuum of line 253.7 mμ, which compared with the sharp edge at 253 mμ is much more pronounced with direct than with alternating current.

Thus when the light of the lamp is irradiated through the spectrograph on the skin no difference is to be expected for the erythematous activity of the lines 302 – 297 mμ with either current. But for the group near 257 mμ a somewhat higher activity might be found with direct current.

The relative energy of the spectral lines falling on the skin

No attempt has been made to measure accurately the absolute intensities of the light falling on the skin. The great variability in sensitivity of human skin makes such measurements of little value (10). Coblentz and Stair (4) indicated a value of 50.10^4 ergs per cm.^{2} at a wave length of 297 mμ for untanned skin to provoke a just perceptible erythema. Hausser and Gauer (11) found 36.10^4 ergs per cm.^{2}, Luckiesh, Holladay, and Taylor (3) 43.10^4 ergs per cm.^{2}, the Council on Physical Therapy proposed 18.10^4 ergs per cm.^{2} (10). A rough estimation yielded under our conditions values not far from that of Coblentz and Stair (4) but the light was falling onto the skin at a sharp angle. Thus with perpendicular irradiation a lower value would have been found.

For these reasons we have refrained from introducing an absolute value for the irradiation doses in our calculations. Instead we have preferred to multiply the irradiation times from Table III with the intensities from Table IV. This can be done in two ways.

The first method is to take both erythemata as a whole, then multiply the sum of the intensities for the wave lengths 302 and 297 mμ and the sum of the intensities of the wave lengths 265, 257, 253 and 248 mμ with their respective irradiation times.

The second method is to use the wave lengths 297 and 257 mμ as representative of both erythemata and, therefore, multiply only the intensities of these two wave lengths with the irradiation doses.

This second way has been chosen for the figures shown in Table V (third and fourth columns).

The values in Table V afford a more correct basis for judging the spectral response of the skin than those of Table III, as the former are then made independent of differences in the distribution of energy radiated by the lamp. This is especially true for the 257 mμ wave length, where the lamp differs significantly when used with the two types of current, making judgment of the spectral response difficult for the values in Table III.

Intermittency effect

Table VI shows the mean values of the MED found in Table V for the two wave lengths considered and for both types of current used. Table VII represents the analogous values but calculated

TABLE VI

Mean values for minimal erythema dose (Table II)

Wave length	Minimal erythema dose		Difference
	Intermittent light	Continuous light	
mμ	sec.	sec.	%
297	1230	1697	38
257	988	1131	14.5

TABLE VII

Mean values for the MED, calculated with the sum of the intensities of all participating spectral lines

Wave length	Intermittent light	Continuous light	Difference
302–297 mμ	3134	4280	37%
265–248 mμ	5261	5281	4%

by following the first way as is indicated in the preceding paragraph. Thus for the values in Table VII, the sum of the intensities of the 302 – 297 mμ lines and the sum of the intensities of the lines 265, 257, 253 and 248 mμ were used.

From the figures of Table VI and VII, it is clear that no significant difference exists between the average MED for the 250 mμ erythema with intermittent or with continuous light.

But the difference in the MED for the 300 mμ group is significantly higher with continuous than with intermittent light. This difference is 37 to 38 per cent, which sharply exceeds the error of ± 20 per cent in the determination of the individual MED. The accuracy in the determination of the mean MED will certainly be lower than 20 per cent, as is shown by the differences in the MED for the 250 mμ erythema in Tables VI and VII.

Therefore, even if the considerations mentioned earlier do not permit us to draw a definite conclusion, the values of Tables VI and VII establish undeniably the influence of intermittent light in provoking the 300 mμ erythema: continuous light requires greater energy and is, therefore, less active for this wave length group than intermittent light.

This effect is absent in the 250 mμ group. Therefore the erythematogenous processes of both wave length groups must be different.

Independence of erythematous responses to both wave length groups

The fifth column of Table V shows a comparison of the MED values for intermittent and continuous light for each person and for both wave length groups. A figure lower than 1 indicates that a smaller amount of energy is needed to provoke the just-perceptible erythema using intermittent light than that needed with continuous light. For the 297 mμ erythema all the figures are lower than 1, in accordance with the conclusion drawn in the preceding paragraph. For the 250 mμ erythema four figures are lower and three higher than 1, thus confirming the absence of a distinct intermittency effect.

In general, the values of the fifth column (Table V) are rather variable for different persons. This variability supports the conclusion drawn above that the processes leading finally to an erythema must be independent of each other for both wave length groups.

Sensitivity of the skin for both wave length groups

The last two columns in Table V show the sensitivities of the seven persons studied. These sensitivities have been calculated by dividing the mean values of the MED given in Table VI by those from columns 2 and 3 in Table V. A figure higher than 1 indicates a higher sensitivity than the mean of these seven persons, a figure lower than 1 the reverse. In Table VIII the sensitivity limits found have been collected.

TABLE VIII

Sensitivity limits found for the seven persons from Table V

Wave length group	Intermittent light	Continuous light
	%	%
300 mμ	0.44–2.67	0.36–3.56
250 mμ	0.46–2.74	0.31–2.66
both groups	0.44–2.74	0.31–3.56

The sensitivity limits are of the order of ⅓–3 times the mean. Our experience over a period of years with a total of some 25 persons for whom the MED has been determined with only intermittent light or for only one wave length group, has given us the impression that the figures in Table VIII may be considered to be the limits of normal sensitivity. A distinct abnormal sensitivity was determined at one time for a person who showed the 297 mμ erythema in approximately 1/50 of the normal time.

Of course statistical compilation of much more material than is given here will be necessary to settle correctly the problem of normal sensitivity for the different wave lengths that can provoke an erythema. It is clear that such a determination will make it possible to ascertain pathological devi-

ations and will offer new possibilities for their better diagnosis.

SUMMARY

A simple method has been described to determine the erythematous response of skin to monochromatic radiation.

The result of measurements for the two main erythemata, *viz.* those provoked by the wave lengths 297 and 257 mμ, indicates the minimum irradiation doses needed to cause these erythemata to be mutually independent.

An undeniable effect of the intermittency of the light used for the irradiation has, moreover, been found for the 297 mμ erythema alone.

The conclusion is therefore drawn that the processes leading to both erythemata must be different.

The variability in sensitivity of human skin has been found to be as great for irradiations with 250 mμ as for those with 297 mμ.

ACKNOWLEDGMENT

I wish to thank Dr. H. Berkelbach van der Sprenkel for his permission to build the spectrograph and for his help with this work.

Also, I wish to thank Dr. J. J. Zoon, Head of the Dermatological Clinic, and his assistants for placing at my disposal several people for measurements.

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Volume 12, No. 2

To be issued May, 1953

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